Flowering Time Studies in Canadian Cultivars and 5-Azacytidine Mutants of Oilseed Flax (*Linum usitatissimum* L.)

A Thesis Submitted to the College of Graduate Studies and Research

In Partial Fulfillment of the Requirements

For the Degree of Master of Science

In the Department of Plant Sciences

University of Saskatchewan

Saskatoon

By

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ABSTRACT

Canada is a global leader in flax production, but flax acreage in Canada remains limited since flax is not well adapted to the northern Prairies. Therefore, breeding early-flowering and early maturing flax cultivars that are adapted to the climate of the northern Prairies is one of the major strategies to expand flax acreage in Canada. The objective of this project is to understand flowering time in flax and generate early flowering genotypes that are adapted to the continental climate of the Canadian Prairies.

This project examined photoperiod sensitivity in five Canadian flax cultivars (CDC Sorrel, CDC Bethune, Flanders, Prairie Thunder and Royal) and three M_9 genotypes derived from 5-azacytidine (5-azaC) treatment (RE1, RE2 and RE3). To investigate how each cultivar or genotype responds to photoperiod changes, a reciprocal transfer experiment between long day and short day conditions was conducted. All cultivars and genotypes were photoperiod sensitive. However, the level of sensitivity and length of the sensitive phase varied among cultivars and genotypes. The five cultivars were more sensitive to photoperiod changes compared with the three mutant genotypes, while RE2, which was the earliest flowering genotype, was the least sensitive genotype.

This project, in addition, examined the expression pattern of *ELF4* (*EARLY FLOWERING 4*), a specific flowering-related gene. This experiment was conducted with three Canadian flax cultivars (CDC Sorrel, CDC Bethune and Royal) and one 5-azaC mutant genotype (RE2). *GAPDH* (Glyceraldehyde 3-phosphate dehydrogenase) was used as a reference gene in RT-qPCR. Results of RT-qPCR demonstrated that CDC Sorrel and CDC Bethune had a similar expression pattern, while Royal and RE2 had a similar expression pattern.

This project also sought to generate early-flowering genotypes by treating CDC Sorrel with 5-azaC as well as to introgress the early-flowering trait from RE genotypes into CDC Sorrel via hybridization. Mutant populations (M_2, M_3, bulk M_3) and hybrid populations (F_2, F_3, and bulk F_3) were grown and evaluated for time to flowering, maturity and height under latitude (53° N) field conditions in 2012 and 2013. 5-azaC treatment did not induce significant differences in flowering or maturity in the CDC Sorrel background. However, the early flowering trait was successfully introgressed into CDC Sorrel background since selected progeny lines flowered significantly earlier than the later flowering CDC Sorrel parental line.

ACKNOWLEDGEMENTS

I would like to express my sincere gratitude to my supervisor Dr. Helen Booker. I appreciate her academic guidance, support, patience, encouragement, and understanding during my studies, as well as all the opportunities she offered me to attend scientific conferences to widen my knowledge and to interact with scientists and students working in the same field from other universities and institutes.

I would also like to thank my supervisory committee members Dr. Graham Scholes, Dr. Ravindra Chibbar, and Dr. Yuguang Bai for their advice, rigorous scientific attitude, professional criticism, assistance, patience and understanding.

Special thanks to Dr. Lester Young and Shannon Froese, Ken Jackle, Barb Boon, and all other staff of flax group at University of Saskatchewan for providing technical and field assistance. Thanks to Dr. Keith Downey for his support and assistance when I decided to apply for University of Saskatchewan, as well as all his kind suggestions for my academic life. I would like to thank Dr. Randy Kutcher and Dr. Fengqun Yu for their kindness, patience and advice.

I would like to thank all my colleagues at the Department of Plant Sciences. I would like to thank my friends Bolin Chen and Bo Gui for their huge support and encouragement which helped me through stressful times. I would also like to thank Kayla Lindenback, Pree Vindhya Edirisinghe, Jihua Xu, Ting Chen, Xue Lin, Wen Zang, Ti Zhang and Tao Zhang for their friendship and help throughout my study period.

I would like to thank Saskatchewan Agriculture Development Fund for my project funding.

Finally, I would like to thank my parents for their unconditional support and care throughout my life, as well as their patience and understanding during my time at University of Saskatchewan.

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5-azaC	5-azacytidine
ALA	Alpha-Linolenic Acid, or α-Linolenic acid
CDC	Crop Development Centre
DTF	Days to flowering
DTM	Days to maturity
DN	Day neutral
HT	Height
HTFB	Height to first branch
KCRF	Kernen Crop Research Farm
LD	Long day
MAD	Modified augmented design
NON	Number of nodes
PS	Photoperiod sensitive
RT-qPCR	Reverse transcriptase quantitative polymerase chain reaction
SD	Short day
SEM	Structural equation model
TSW	Thousand seed weight

CHAPTER 1

1. Introduction

Flax (*Linum usitatissimum* L.) is an ancient crop that belongs to the genus Linum of the family Linaceae. Originally adapted to Europe and Asia, flax is now grown in different areas worldwide (Casa et al., 1999; Berti et al., 2010). In Canada, flax is produced mainly in southeast Saskatchewan and southwest Manitoba.

Although Canada is a leading flax producer in the world, flax acreage in Canada is limited since it is not well adapted to the northern Prairies (Figures 1.1 and 1.2). Traditionally, flax in Saskatchewan is adapted to southern and central areas, where the first fall frost generally ranges from September 9th to 23rd each year (Figure 1.2). To expand flax acreage in Canada, one strategy is to breed new flax cultivars that are better adapted to the northern Prairies.

There are three major challenges in breeding northern-adapted flax cultivars. First and foremost is the short growing season. Frost-free days in the far northern grain belt of Saskatchewan are relatively limited, ranging from only 85 to 95 days, while flax requires 90 to 150 days to reach maturity (Diederichsen and Richards, 2003; Bueckert and Clarke, 2013). The second challenge is the vulnerability of flax plants to frost and cold injury. The average first fall frost in the Saskatoon area occurs in mid-September (Figure 1.2). If flax does not mature before the first fall frost, both its seed yield and seed quality is adversely influenced. The third challenge is the reduction in grain seed yield. Earliness in flowering and maturity often leads to a reduction in grain yield since early flowering is often associated with a shorter vegetative stage, which will further result in low grain yields for many crops.

Figure 1.1 Traditional flaxseed growing areas in western Canada (source: Flax Council of Canada).

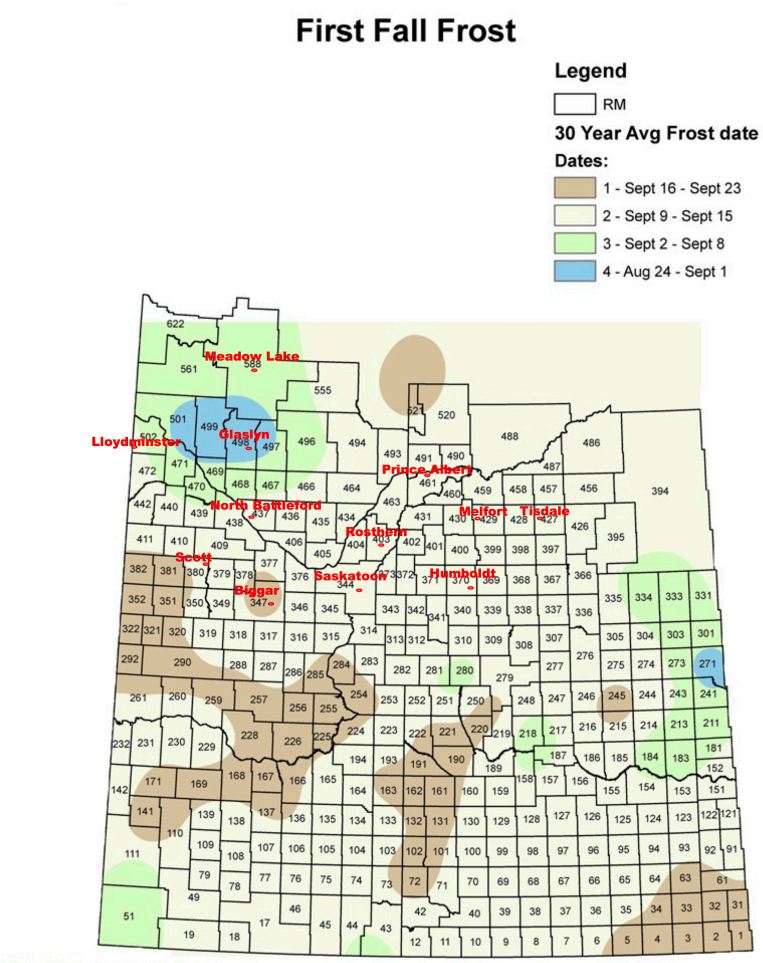


Figure 1.2 Thirty-year first fall frost date in Saskatchewan (adapted from the Saskatchewan Crop Insurance Corporation, 2014).

The climate in the northern Prairies features a risk of early fall frost, the probable timing of which in central Saskatchewan ranges from August 24th to September 1st for Glaslyn (53.358°N); September 2nd to 8th for Meadow Lake (54.1242°N) and Lloydminster (53.2783°N); and September 9th to 15th for North Battleford, Scott, Rosthern, Saskatoon, and Melfort (range 52.1333°N to 52.8564°N) (Saskatchewan Crop Insurance Corporation, 2014). Given the risk of early frost affecting seed quality, it is especially important to develop early-maturing cultivars for the conditions experienced in the northern Prairies.

However, in a structural equation model (SEM) that was used to identify major traits that are significantly associated with flax grain yield, no significant relationship between days to flowering (DTF) and yield or days to maturity (DTM) and yield was found in the Canadian flax core collection grown at Kernen Crop Research Farm (KCRF), Saskatoon, SK (Zhang et al., 2014). FP2385 is an advanced breeding line developed at University of Saskatchewan. It is adapted to the northern Prairies and received support for registration in 2014 (Booker, 2014). FP2385 is early maturing (equal to the check cultivar Prairie Thunder) while yielding 6% more than the check cultivar CDC Bethune, the yield standard. Thus, breeding for earlier flowering/maturity flax cultivars without adversely influencing its grain yield can be realized. Breeding early flowering and early maturing flax cultivars that are adapted to the northern Prairies can protect the flax crop from frost damage, which would benefit Saskatchewan producers.

There are three major benefits to breeding northern-adapted flax cultivars. Firstly, it increases the acreage where flax can be successfully grown by shortening the life cycle. Thus, the short growing season in the northern prairies will no longer limit flax production in Canada. Secondly, growing flax under cooler climate of the northern Prairies can improve alpha-linolenic acid (ALA) levels and decrease saturated fatty acids level in flaxseed. It was reported that flax varieties grown under a long photoperiod and cool temperatures have high oil content, linolenic acid content and iodine number (Sosulski and Gore, 1964). Thirdly, for flax adapted to the northern Prairies earlier flowering and maturity can improve flaxseed quality by reducing levels of green seeds as well as frost-damaged seeds.

There are three hypotheses for this study:

1. Long day conditions accelerate flowering time in flax, and different flax cultivars and genotypes respond will differently to day length changes.

2. *ELF4* expression pattern is related to flowering time in flax.

3. The early flowering trait can be introgressed into CDC Sorrel from early flowering genotypes via hybridization, and will be stable under field conditions.

This study focuses on three objectives:

1. To examine photoperiod sensitivity in different flax cultivars and genotypes. Photoperiod plays an important role in the regulation of flowering time. Understanding photoperiod sensitivity of different flax cultivars and genotypes is the first step to understand flowering time in flax.

2. To examine the expression pattern of a flowering related gene *ELF4* (*EARLY FLOWERING 4*). *ELF4* was first studied in *Arabidopsis thaliana*, where it was found to inhibit floral transition. Three orthologues of *ELF4* were identified in flax. Examining the expression patterns will help to understand flax flowering time on a molecular level.

3. To introgress earliness from early-flowering flax genotypes into CDC Sorrel by crossing and identifying early-flowering variants from 5-azacytidine (5-azaC) treated CDC Sorrel. Three 5-azaC derived genotypes produced by Dr. M. A. Fieldes (1994) were used in this study. The offspring populations were tested under field conditions at KCRF, Saskatoon, Saskatchewan.

CHAPTER 2

2. Literature Review

2.1 Flax and its production in Canada and in the world

Flax (*Linum usitatissimum*. L.) (Figure 2.1) is one of the earliest domesticated crops in human history. As an ancient crop, there is a large genetic diversity in flax. Flax is a diploid (2n=30) crop plant and a self-pollinator. In the latest proposed classification by Čaernomorskaja and Stankevidč, flax is classified into five taxonomic groups (Table 2.1) (Muir and Westcott, 2003). Flax is grown for seed (linseed) or fibre (isolated from the stem of the plant). Oilseed flax (linseed flax) is shorter than fibre flax, and has larger seeds with a more branched growth habit (Nichterlein and Horn, 2005). In different areas, different flax cultivars are cultivated with different local climates and growing methods (Muir and Westcott, 2003). For example, flax is cultivated under short-day environments in subtropical areas. However, in temperate climates, flax is cultivated as a summer annual crop where time to maturity (after seeding) can vary from 90 to 150 days (Figure 2.2) (Diederichsen and Richards, 2003; Bueckert and Clarke, 2013).

Humans have been cultivating flax for thousands of years. Since ancient times, flax has been valued for the dual purpose of its oil and fibre (Muir and Westcott, 2003). In recent years, much attention was drawn to its functional food components such as dietary fibre and ALA, as oilseed flaxseed is a rich resource for two essential fatty acids including alpha linolenic acid (ALA) and linoleic acid (LA) (Jhala and Hall, 2010). ALA level in other oilseed crops is relatively lower comparing with flaxseed. For example in

Figure 2.1 A flowering flax plant (Howard, 1924).

Adapted from “Flax the genus linum” (Muir and Westcott, 2003)

Table 2.1 The latest proposed classification by Čaernomorskaja and Stankevidč (1987).

English name	Main characteristics	Formal name according to Čaernomorskaja and Stankevidč (1987)	Placement in the system of Kulpa and Danert (1962)
Fiber flax	One stem; tall	Ssp. *usitatissimum*	Convar. *elongatum*
Intermediate flax	One stem; medium height	Ssp. *Intermedium* Czernom.	Convar. *usitatissium*
Crown flax	Several stems; short; late mature	Ssp. *humile* (Mill.) Czernom.	Convars. *Mediterraneum* and *usitatissimum*
Large-seeded flax	Thousand seed weight larger than 7 g	Ssp. *latifolium* (L.) Stankev.	Convar. *Mediterraneum*
Semiwinter flax	Prostrate growth habit	Ssp. *bienne* (Mill.) Stankev.	Convar. *usitatissimum*

Convar. *elongatum,* convar. *usitatissium,* and convars. *mediterraneum* describe typical fibre flax, intermediate/ dual purpose flax, and large seeded flax (used for seed production only), respectively. Adapted from "Flax the genus linum" (Muir and Westcott, 2003).

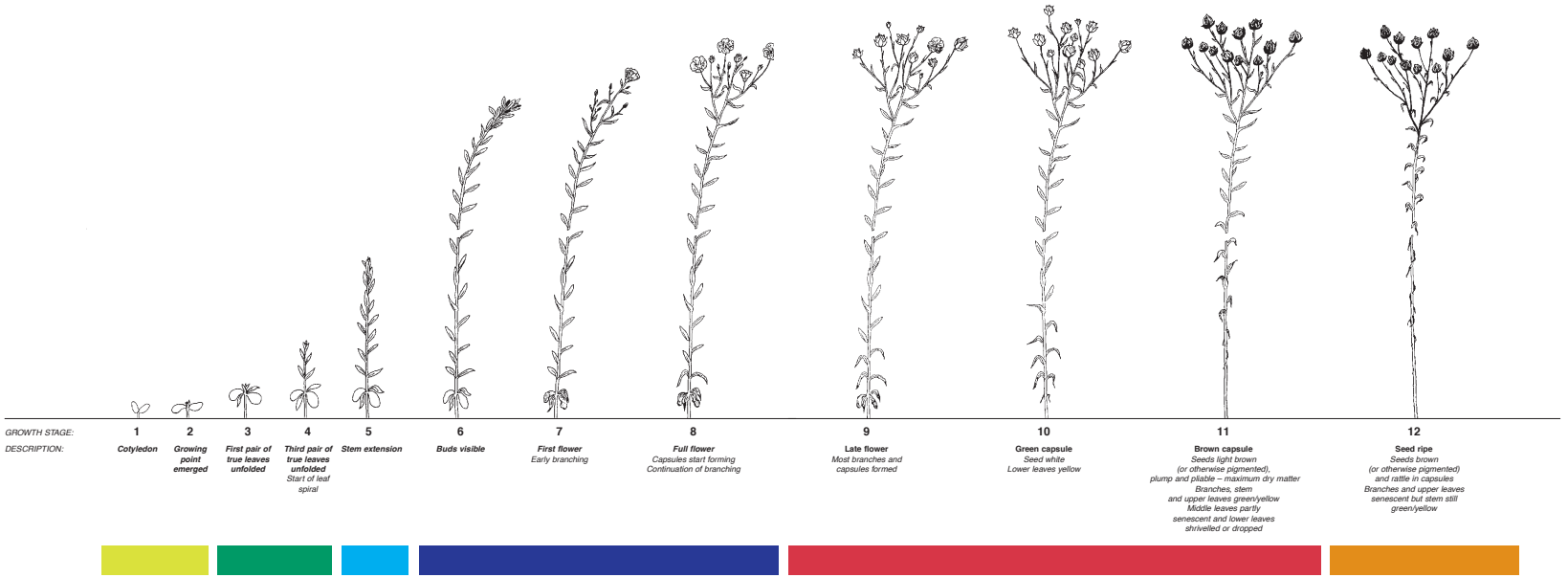


Figure 2.2 Flax main stem growth stages (Flax Council of Canada, 1996).
Flax growth cycle 90-150 days from seeding to maturity. Generally there are three stages: vegetative 1-5, flowering 6-10, and maturity 11-12.

canola oil ALA makes up about 11% of the fatty acids and in soybean oil it is about 7-8% (Messina, 1999; Lin et al., 2013). On average, ALA account for 57% of the total fatty acid in flaxseed of Canadian cultivars (Jhala and Hall, 2010). Dietary fibre in flaxseed can contribute to the reduction in the blood total cholesterol and glucose levels, while its ALA can contribute to the reduction of inflammation and risk of stroke (Muir and Westcott, 2003). In fact, it was found that, the consumption of flaxseed is associated with the reduction in the occurrence of breast cancer (Lowcock et al., 2013).

According to the Flax Council of Canada, Canada is currently the world's largest flax producer (about 25% of total world production) and exporter (FAO, 2014), while Saskatchewan is the largest flax-producing region in Canada. In 2014, 635, 300 hectares of land in Canada was seeded with flax (Statistics Canada, 2014).

In Canada flax is mainly grown in the Prairie Provinces for seed oil (linseed flax). In the last ten years (2004-2013), flax production in Western Canada fluctuated between 398,900 tonnes (2011-2012) and 1,082,000 tonnes (2005-2006). Following a sharp decrease in the year 2010-2011, flax production has increased progressively year by year (Figure 2.3).

2.2 Frost and cold injuries to crops

In Canada, early maturity is required for flax due to the short growing season, which can protect the crop from various biotic and abiotic stresses, such as frost, disease, seasonal heat, and drought.

Frost and low temperature are among the most common abiotic stresses that lead to freezing injury and result in crop loss (Mayland and Cary, 1970; Burke et al., 1976; Pareek et al., 2010). Frost injury happens in individual plant cells when cell wall water

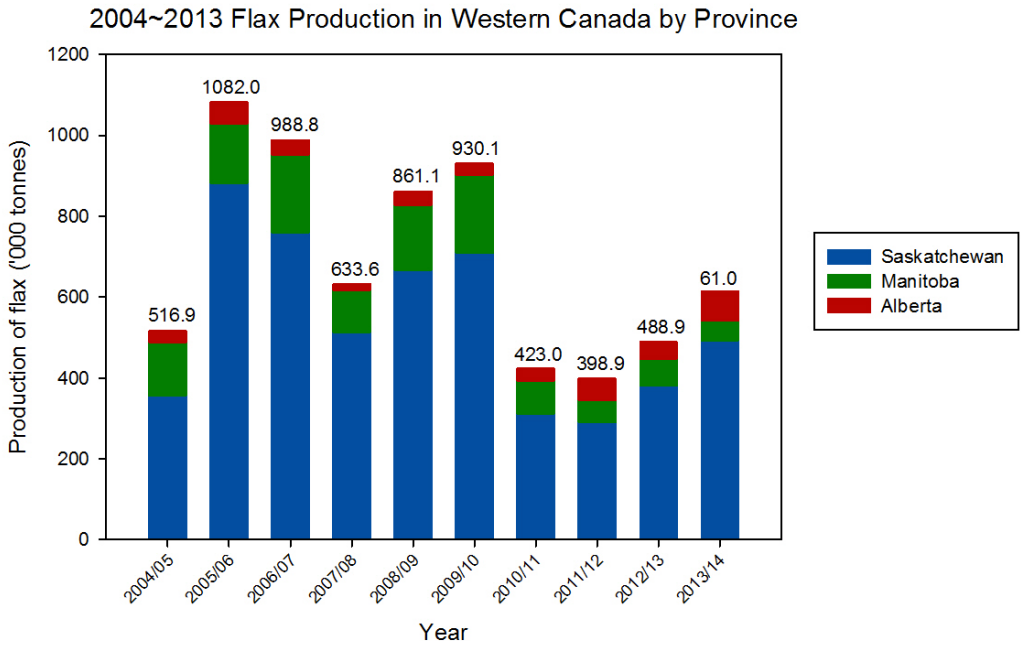


Figure 2.3 2004-2013 Flax production in Western Canada by province (Flax Council of Canada, 2014).
Blue bars represent flax production in Saskatchewan, the main flax producing area in western Canada. Green bars and red bars represent flax production in Manitoba and Alberta, respectively.

either inside or outside (intracellular or extracellular) freezes and the ice crystals puncture plant cell walls (Mayland and Cary, 1970). Several factors can affect the degree of frost damage including temperature, moisture content of plants, duration of the frost the speed to reach detrimental temperature, plant maturity, plant health, and exposed parts of plants under freezing temperatures (Phelps, 2004).

Frost can affect crops in many different ways. For cereal crops, frost can both decrease their yields and reduce seed viability of the exposed seeds. For oil crops, such as canola and mustard, frost can not only reduce yields, but also affect their oil quality significantly (Saskatchewan Ministry of Agriculture, 2008). For flax, depending on the length of exposure, immature seeds can be severely damaged or even destroyed by temperatures from 0-4°C, turning seeds black (Phelps, 2004). In addition, flax is especially vulnerable to frost, since the growing point of a flax seedling is completely exposed above ground (Anonymous, 2002).

The short growing season in Saskatchewan makes it difficult for the flax crop to mature in this region. Cultivated flax usually takes 90 to 150 days to mature (Diederichsen and Richards, 2003). However, the frost-free days in Saskatchewan range from 85 to160 days, and growing season in far northern Saskatchewan lasts only 85 to 95 days (Figure 1.2) (Government of Canada, 2010).

2.3 The influence of photoperiod on flowering time

The regulation of flowering is very important in crop development. Many environmental factors affect flowering time in plants, such as photoperiod, light quality and quantity, temperature, vernalization, nutrition and water availability (Thomas and Vince-Prue, 1996; Levy and Dean, 1998; Ausín et al., 2005; Bernier and Périlleux, 2005;

Munir et al., 2010). One of the most important exogenous factors that has a major effect on flowering time is photoperiod and seasonal changes in photoperiod (Jung and Muller, 2009).

Photoperiod is the recurring cycle of light and dark periods, which is crucial to different types of organisms (animals, plants and fungi). It regulates a number of developmental responses. For example, a plant can adjust itself to a particular environmental condition based on photoperiod changes (Jackson, 2009).

According to different photoperiod responses, plants can be divided into three major types: 1) short-day (SD) plants, which only flower normally when the photoperiod is shorter than its critical day length, such as rice, soybean, sorghum, maize, and chrysanthemum; 2) long day (LD) plants, which only flower when the photoperiod is longer than its critical day length, such as spring wheat, barley, and opium poppy, and snapdragon; and 3) day-neutral (DN) plants, which do not respond to photoperiod, such as tomato, cucumber, and pepper. Generally, LD conditions delay flowering time or even fail to induce flowering in SD plants, while promoting flowering in LD plants; and vice versa for SD conditions.

Experiments have been conducted to study photoperiod sensitivity in several types of plants by using reciprocal transfers under controlled environments: soybean [*Glycine max* L.] (Ellis et al., 1992), rice (*Oryza sativa* L.) (Collinson et al., 1992), sorghum [*Sorghum bicolor* L. *Moench*] (Ellis et al., 1997; Alagarswamy et al., 1998), opium poppy (*Papaver somniferum.* L.) (Wang et al., 1997), chrysanthemum (Adams et al., 1998), and snapdragon (*Antirrhinum Majus* L.*)* (Munir et al., 2010).

Sufficient data indicates that cultivated flax is a LD plant since a SD condition delays the onset of its reproductive stage (Sizov, 1955; Domantovich et al., 2012). However, there is little description in the literature on flax's response to photoperiod (Domantovich et al., 2012). Thus, assessing the length of different stages of photoperiod sensitivity in flax cultivars and genotypes is one of the objectives in this study, which offers a better understanding of genetic control of flowering time in flax.

2.4 Photoperiod sensitivity and the Circadian clock in Arabidopsis

Light has the most significant influence in the growth and development of a flax plant compared with other environmental factors including temperature, gravity, and water mineral availability. In a plant, leaves perceive photoperiod, and the growing point on the shoot apex is induced to flower (Pan, 2001c). Photoreceptor is the substance that responds to light (Yong et al., 2000). After the receptor intercepts a light, the signal is then transported through phloem to the apical shoot tips, where the response to the photoperiod is takes place (Yong et al., 2000). Phytochrome is the most-studied photoreceptors in plants, located in different plant organs, with the highest concentration in apical meristems and root tips (Pan, 2001b). In a plant cell, phytochrome spreads out in the membrane system, cytosol, and nucleus. There are two types of phytochrome: a red light absorbing form Pr and a far-red light absorbing form Pfr. Pr transfers to Pfr after absorbing 660 nm red light; Pfr transfers to Pr after absorbing 730 nm far-red light (Pan, 2001b). In Arabidopsis, at least five genes with different functions were found to be responsible in coding for phytochrome proteins. They are: *PHYA*, *PHYB*, *PHYC*, *PHYD*, *PHYE* (Pan, 2001b). *PHYA* codes for type I phytochrome (Phy I, absorption peak 666 nm), which absorbs 700-750 nm far red light and breaks down if exposed to light. The

other four genes code for type II phytochrome (Phy II, absorption peak 652 nm) that absorbs 600-700 nm red light remaining stable when exposed to light.

Photoperiod sensitivity in plants is closely related to the circadian clock. For instance, CO (CONSTANS) is an important transcription factor in regulating pathways in Arabidopsis. The regulation of CO is at the transcriptional level by several genes that are involved in the circadian clock control, and at the protein level by photoreceptors (phytochromes and cryptochromes) that stabilize or de-stabilize CO (Jung and Muller, 2009).

The circadian clock allows plants to sense day/night change rhythms during the 24 h cycle. It is in general composed of three parts: an input pathway, a central oscillator(s) and an output pathway (Kikis et al., 2005; Wang et al., 2011).

In the long history of studying the circadian clock in plants, *Arabidopsis thialiana*, an important model plant widely used in genetics and molecular biology, displays circadian rhythm in many living activities during its life cycle. For example, circadian rhythm was found in its cotyledon and leaf movement, elongation rate of inflorescence, hypocotyl, abaxial petiole cells, and adaxial petiole cells (McClung, 2006b).

Figure 2.4 illustrates the basic molecular model of the circadian clock in *Arabidopsis thaliana*. Three interlocked feedback loops constitute the central oscillator, which is the major part of the model (Figure 2.4). CIRCADIAN AND CLOCK ASSOCIATED1 (CCA1) and LATE ELONGATED HYPOCOTYL (LHY) are two single Myb domain transcription factors, both of which are involved in all three loops. Among the three loops, CCA1/LHY/ Timing of CAB expression1 (TOC1) is the core feedback loop.

2.5 The role of the *ELF4* in flowering time regulation

In the life cycle of a plant, floral transition is one of the major phase changes. To establish this transition, phytochome movement in the phloem of a plant initiates the signal to respond to changes in day length. Similar to flax, *Arabidopsis thaliana* flowers earlier under LD conditions than under SD conditions, which indicates that it is a LD plant.

Several genes are involved in regulating flowering time. Among them, *ELF4* is an important one that controls photoperiod perception and circadian management (Doyle et al., 2002). It was confirmed that *ELF4* is closely related to the *CCA/LHY-TOC1* feedback loop (McWatters et al., 2007). It is also necessary for the *CCA1* and *TOC1* expression under a free-running condition (constant light/dark condition). Overexpression of *ELF4* in plants delays flowering time and results in a longer circadian period (McWatters et al., 2007).

It was also found that rhythmicity in *ELF4* mutants (*elf4*) was disturbed under a free-running condition (Doyle et al., 2002; Kikis et al., 2005; McWatters et al., 2007). Moreover, *elf4* mutant plants exhibited early flowering under a non-inductive photoperiod (Doyle et al., 2002).

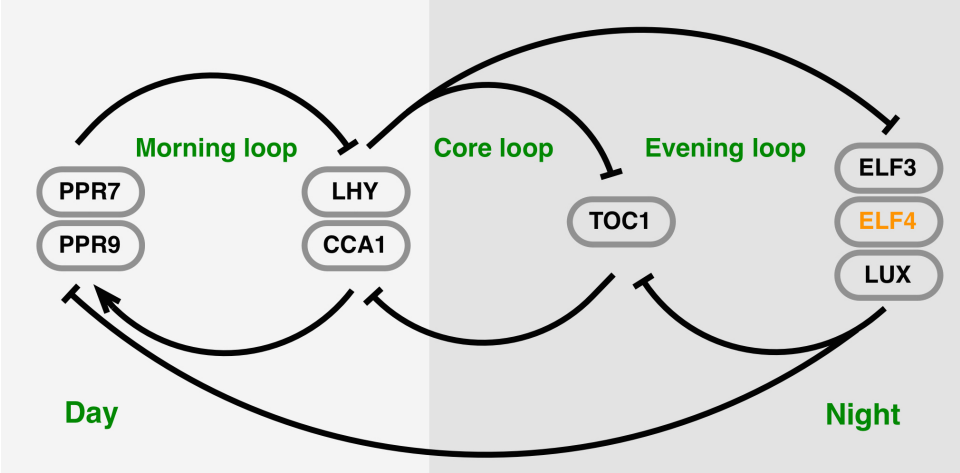


Figure 2.4 A simplified molecular model of the *Arabidopsis thaliana* circadian oscillator. CCA1, LHY and TOC1 consist the core loop, in which CCA1 and LHY are Myb-type transcription factors, and TOC1 is the Pseudo response regulator. The core loop connects the morning and the night loops. In the morning loop, the expression of *Pseudo-Response Regulator* genes (*PRR*s) is activated by CCA1 and LHY, which in turn inhibits the expression of *CCA1* and *LHY*. In the night loop, ELF3, ELF4 and LUX consist a protein complex, which restrains the expression of *PRR9*.
Adapted from "Plant circadian rhythms" (McClung, 2006b), "The circadian clock goes genomic" (Staiger et al., 2013), and "Tissue-specific clocks in Arabidopsis show asymmetric coupling" (Endo et al., 2014).

2.6 DNA methylation in plants

DNA methylation is an important regulatory mechanism of gene expression, by activating or silencing transcription (Furner et al., 1998; Matzke and Matzke, 1998; Scheid et al., 2002).

DNA methylation in plants is an example of epigenetic control. It can induce changes in gene expression without altering the primary DNA sequence (Fieldes, 1994). However, under some circumstances, alterations in DNA methylation may regulate or induce genetic changes in plants (Finnegan et al., 1996; Richards, 1997). Chemical treatment is one of the methods that induce demethylation or hypomethylation in DNA.

2.7 5-azacytidine treatment of plants

5-azacytidine (5-azaC, $C_8H_{12}N_4O_5$) is a potent growth inhibitor and cytotoxic agent (Jones, 1984). It can cause DNA demethylation or hemi-demethylation through the inhibition of DNA methyltransferase. The inhibition of DNA methyltransferases by 5-azaC means that cytosine residues incorporated into DNA during replication remain unmethylated (Takeno, 2010). Typically demethylated DNA is transcriptionally active (Bird, 2002).

In addition to demethylating DNA, 5-azaC also functions as a weak mutagen, directly influencing DNA replication and protein synthesis (Jones, 1984; Brown, 1989).

Treatment with 5-azaC has induced heritable changes in *Oryza sativa* (Sano et al., 1990; Akimoto et al., 2007), *Triticale* (Heslop-Harrison, 1990; Amado et al., 1997), *Brassica oleracea* (King, 1995), *Nicotiana tabaccum* (Vyskot et al., 1995), *Melandrium album* (Janoušek et al., 1996), and *L. usitatissimum* (Fieldes and Harvey, 2004).

Treating germinating flax seeds with 5-azaC induced several early flowering genotypes (Fieldes, 1994; Fieldes and Amyot, 1999). DNA hypomethylation was observed five to nine generations beyond the treatment generation (Fieldes and Harvey, 2004). Some early flowering genotypes induced by 5-azaC treatment flowered 7-13 days earlier than the untreated controls, which resulted from a shortened vegetative stage (Fieldes and Harvey, 2004). Flowering time was more variable among the first progeny of the treated plants (Fieldes, 1994).

2.8 Royal and RE genotypes

The first 5-azaC treated early flowering genotypes (RE1, RE2 and RE3) (Table 2.2) were induced from an old flax cultivar "Royal", which was bred by plant breeders in Department of Field Husbandry (now Plant Sciences) of University of Saskatchewan and distributed in 1939. Royal became a popular cultivar in the 1940's (Canada Department of Agriculture and McGregor, 1953). Currently Royal is no longer used as newer flax cultivars with better agronomic characteristics and better adaption to the climate have been released.

RE genotypes are early-flowering hypomethylated mutant genotypes induced by 5-azaC treatment of germinating seeds of Royal (Fieldes et al., 2005). One of the mutant genotypes, RE2, accumulates more *LEAFY* (*LFY*) transcript in tissues of the main stem shoot tips at earlier stage in development than the control genotypes Royal (De Decker, 2007). *LFY* is one of the identified integrator genes that integrate the signals from all flowering time genes and pathways to establish flowering time (House, 2010). Over expression of *LFY* leads to a truncated adult stage, which was observed in early-flowering Arabidopsis (House, 2010). In Arabidopsis, there are two flowering time genes that are

affected either directly or indirectly by methlyation. Altered methylation at the *VERNALIZATION2 (VRN2)* locus is thought to regulate expression of *FLOWERING LOCUS C (FLC)*, leading to a decrease in expression, which, in turn, causes early flowering in vernalization-sensitive ecotypes of Arabidopsis (Finnegan et al., 2000; Genger et al., 2003). Conversely, a direct demethylation effect at the *FLOWERING WAGENINGEN* (*FWA*) locus delays flowering time in the Landsberg erecta and Columbia ecotypes of Arabidopsis (Genger et al., 2003).

2.9 CDC Sorrel, CDC Bethune, Flanders and Prairie Thunder

Four cultivars (CDC Sorrel, CDC Bethune, Flanders and Prairie Thunder) were selected for this project based on their time to flowering and relative maturity rating as described in the variety descriptions (Rowland et al., 1990; Rowland et al., 2002; Government of Canada, 2008; Government of Canada, 2009) (Table 2.2). Flanders, CDC Bethune and Prairie Thunder are check cultivars utilized in the linseed flax co-operative trials (Table 2.2). CDC Sorrel and CDC Bethune are late maturing cultivars that currently account for 2/3 of the total flax acreage. Flanders is a late maturing cultivar used as the check cultivar in the Brown and Yellow Linseed Corporative Test. Prairie thunder is a medium maturing cultivar, utilized in the Northern Linseed Corporative Test as the check cultivar for maturity.

Table 2.2 Cultivars and genotypes used in this project.

Cultivars/ Genotypes	Pedigree	Year of Release	Relative Maturity Description	Variety Description
CDC Sorrel	FP956/Vimy	2008	Late	Popularly grown Canadian flax cultivars; account for 2/3 of total flax acreage
CDC Bethune	NorMan/FP857	1998	Medium-Late	
Flanders	McGregor/Dufferin	1989	Late	Check cultivar for maturity in the Brown and Yellow Linseed Cooperative Test
Prairie Thunder	FP974 / FP1043	2009	Early	Check cultivar for maturity in the Northern Linseed Cooperative Test
Royal	-	1939	Medium	Old oilseed variety (1940s)
RE 1	Royal	-	Early	5-azacytidine treated early flowering derivative genotypes
RE 2	Royal	-	Early	
RE 3	Royal	-	Early	

CHAPTER 3

3. Photoperiod sensitivity of Canadian flax cultivars and 5-azacytidine treated early flowering derivatives

3.1 Abstract

This study examined the timing of the photoperiod-sensitive phase in selected flax cultivars and genotypes and assessed the length of different stages of photoperiod sensitivity. The material examined included five flax cultivars (CDC Bethune, CDC Sorrel, Flanders, Prairie Thunder, Royal) and three 5-azaC treated early flowering derivatives of the cultivar Royal (RE1, RE2, and RE3).

Weekly reciprocal transfers were conducted between long day (LD) and short day (SD) controlled environments. Observations were made on days to flowering (DTF), node number (NON), plant height (HT) and height to first branch (HTFB).

All the cultivars and genotypes tested were photoperiod sensitive and exhibited reduced time to flowering upon transfer to the LD environment. The timing of the photoperiod-sensitive phase and the length of different stages differed among cultivars and genotypes. HT and HTFB were reduced in plants grown under a LD environment as compared to a SD environment, suggesting that growth under LD limits the vegetative stage, reduces the time to flowering and extends the reproductive phase in the selected flax cultivars and genotypes.

The response to photoperiod changes varied among cultivars and genotypes. The early flowering RE1, RE2 and RE3 genotypes exhibited a significantly truncated vegetative phase and earlier flowering than the cultivars under LD conditions. Cultivars CDC Sorrel, CDC Bethune, Prairie Thunder, Flanders and Royal were more photoperiod sensitive compared with, RE1, RE2 and RE3.

The magnitude of the influence of photoperiod on time to flowering differed among all cultivars and genotypes studied. RE2 was the least photoperiod sensitive genotype followed by RE3, RE1, Flanders, CDC Bethune, Royal, Prairie Thunder and CDC Sorrel.

3.2 Introduction

Cultivated flax is considered a LD plant, where lengthening days hastens the reproductive phase of development. The degree of photosensitivity in flax varies greatly (Zhang, 2013). In a study that characterized early and late flowering flax accessions conducted under both controlled and field conditions, it was also found that accessions differed in photoperiod response (Zhang, 2013).

Photoperiod changes are consistent from year to year and provide a signal to crop plants of approaching environmental alterations and the requirement to transition from vegetative to reproductive growth (Song et al., 2013). Photoperiod sensitivity is an important factor that influences crop phenology. Understanding photoperiod sensitivity in flax provides the knowledge base to predict flowering and maturity times in this crop species. Thus, characterizing photoperiod sensitivity for flax is important for plant breeders to develop cultivars adapted to the short frost-free growing season in the northern Prairies.

Reciprocal transfer experiments have been conducted to quantify the vegetative, photoperiod sensitive and reproductive phases of development in different crops. In a reciprocal transfer experiment, there are two controlled environments that have different settings such as day/night temperature or day length. These parameters are assumed to affect the growth and development of a plant. By exchanging plants between these two environments, observations can be made on characteristics which are influenced such as

flowering time and plant height. In a reciprocal transfer experiment conducted on four rice cultivars (*Oryza sativa L.*), which is a short day plant, panicle initiation was delayed for 11-14 days under LD conditions at both warmer (32/26 °C) and cooler (28/20 °C) day/night temperatures (Collinson et al., 1992). In soybean [*Glycine max (L)*] which is a SD plant, the maximum difference in days to flowering between LD-SD transfer and SD-LD transfer was 54 days (Ellis et al., 1992).

Yin (2008) proposed a model to analyze reciprocal-transfer studies conducted on three rice (*Oryza sativa*) cultivars to estimate the length of each stage (basic vegetative phase, BVP; photoperiod sensitive phase, PSP; and post photoperiod sensitive phase, PPP) that had different responses to high and low temperatures. This was a new approach that allowed researchers to quantify significant impacts of the environment on plant growth and development. Similar to temperature, day length is another important parameter that influences plant development, and therefore it is suitable to apply this model to analyze photoperiod responses of flax and assess the length of pre-sensitive (vegetative), photoperiod sensitive (transition) and post-sensitive stages.

In this study, a reciprocal transfer experiment under controlled environments was conducted:

a) To examine the timing of the photoperiod-sensitive phase in different flax cultivars and genotypes;
b) To assess the length of different stages of photoperiod sensitivity of flax cultivars and genotypes.

3.3 Materials and methods

3.3.1 Original germplasm

Five cultivars (CDC Sorrel, CDC Bethune, Flanders, Prairie Thunder and Royal) and three mutant genotypes (RE1, RE2 and RE3) were selected for this experiment based on their time to flowering and relative maturity rating as described in the variety descriptions (Canada Department of Agriculture and McGregor, 1953; Rowland et al., 1990; Rowland et al., 2002; Government of Canada, 2008; Government of Canada, 2009) (Table 2.2).

3.3.2 Chamber settings and transfer plan

Temperature settings were under 22/16 ºC (12/12 h), which were the same for both LD and SD growth chambers. Plants under LD conditions were grown at 16/8 h photoperiod and 300 μmol photons $m^{-2}s^{-1}$. Plants under SD conditions were grown under identical conditions but with a 10/14 h photoperiod.

There were three plants in each pot and 15 pots for each cultivar or genotype. Starting from the 11th day after seeding, six plants (two pots) were transferred weekly from one chamber to the other. Transfers were conducted six times with seven days intervals between every transfer. The remaining nine plants (three pots) in each growth chamber were used as control pots for each cultivar or genotype. In total there were 120 pots (360 plants) in each growth chamber.

3.3.3 Experimental design

The experiment was conducted in a completely randomized design (CRD). The positions of the pots were randomly assigned within each cart in each growth chamber weekly. The entire experiment was repeated once.

3.3.4 Data collection for each individual plant

Observations were taken on days to emergence, days to flowering after seeding, number of nodes from bottom of the plant to branching point (NON), plant height (HT) and height from bottom of the plant to the branching point (height to first branch, HTFB) for each plant. Days to flowering (DTF) was obtained by deducting days to emergence from days to flowering after seeding. HT and HTFB were measured after flowering, and NON were counted after plants were dried.

3.4 Data analyses

Flowering time was averaged among six plants of each time point (six replications) for each cultivar or genotype. Tukey's tests were conducted to compare DTF for different treatments. The length of different stages was quantified using the model proposed by Yin (Yin, 2008), which was initially used to analyze lengths of different phases that responded to high and low temperature conditions. This model can also be applied to other environmental factors that significantly affect plant growth and development (Yin, 2008).

By applying Yin's model, the length of each sub-phase (BVP, PSP, and PPP) was calculated as follows (Figure 3.1) (*f*: from sowing to flowering; *t*: from sowing to transferring):

$f_L = I_{1L}+I_{2L}+I_{3L}$

$f_S = I_{1S}+I_{2S}+I_{3S}$

For the plants transferred from SD to LD, we could obtain the coordinate of the point 'A' as:

A: $(0, f_L)$.

For point B, the *t*-axis were obtained as I_{1S}. For its *f*-axis, the difference in flowering time between A and B was caused by the difference in the first sub-phase (BVP) between LD and SD, which equalled to I_{1S}-I_{1L}. Thus, the coordinate of the point 'B' was:

B: (I_{1S}, f_L+I_{1S}-I_{1L})

By using the coordinate of those two points, were calculated the relationship of *f* and *t* as $f = I_{1L} + I_{2L} + I_{3L} + (1\text{-}I_{1L}/I_{1S})t$ for all $t \leq I_{1S}$.

Similarly, the difference in flowering time between B and C was caused by the difference in the second sub-phase (PSP) between LD and SD, which equaled to I_{2S}-I_{2H}. Thus, the coordinate of the point 'C' was:

C (I_{1S}+I_{2S}, f_L+I_{1S}-I_{1L}+I_{2S}-I_{2L})

And the coordinate of the point 'D' was:

D (f_S, f_S)

Hence, for plants transferred from SD to LD:

$$
\begin{aligned}
f &= I_{1L} + I_{2L} + I_{3L} + (1\text{-}I_{1L}/I_{1S})t && \text{if } t \leq I_{1S} \\
f &= I_{2L} + I_{3L} + I_{1S} + (1\text{-}I_{2L}/I_{2S})(t - I_{1S}) && \text{if } I_{1S} < t \leq I_{1S} + I_{2S} \\
f &= I_{3L} + I_{1S} + I_{2S} + (1\text{-}I_{3L}/I_{3S})(t - I_{1S}\text{-}I_{2S}) && \text{if } I_{1S} + I_{2S} < t \leq f_S \\
f &= I_{1S} + I_{2S} + I_{3S} && \text{if } t > I_{1S}+I_{2S}+I_{3S}
\end{aligned}
\qquad (1)
$$

Similarly, for the transfer from LD to SD, the coordinate of points 'E', 'F', 'G' and 'H' were:

E(0, f_S)

F(I_{1L}, f_S+I_{1L}-I_{1S})

G(I_{1L}+I_{2L}, f_S+I_{1L}-I_{1S}+I_{2L}-I_{2S})

H(f_L, f_L)

And the relationship between f and t from LD to SD were:

$$
\begin{aligned}
&f = I_{1S} + I_{2S} + I_{3S} + (1 - I_{1S}/I_{1L})t && \text{if } t \le I_{1L} \\
&f = I_{2S} + I_{3S} + I_{1L} + (1 - I_{2S}/I_{2L})(t - I_{1L}) && \text{if } I_{1L} < t \le I_{1L} + I_{2L} \\
&f = I_{3S} + I_{1L} + I_{2L} + (1 - I_{3S}/I_{3L})(t - I_{1L} - I_{2L}) && \text{if } I_{1L} + I_{2L} < t \le f_L \\
&f = I_{1L} + I_{2L} + I_{3L} && \text{if } t > I_{1L} + I_{2L} + I_{3L}
\end{aligned}
\qquad (2)
$$

Then two dummy variables were introduced:

Z_0=1 and Z_1=0 for plants transferred from SD to LD,

Z_0=0 and Z_1=1 for plants transferred from LD to SD.

Thus equation (1) and (2) above can be represented collectively as:

$$f = Z_0[I_{1L} + I_{2L} + I_{3L} + (1 - I_{1L}/I_{1S})t] + Z_1[I_{1S} + I_{2S} + I_{3S} + (1 - I_{1S}/I_{1L})t]$$

if $t \le Z_0 I_{1S} + Z_1 I_{1L,}$ or

$$f = Z_0[I_{2L} + I_{3L} + I_{1S} + (1 - I_{2L}/I_{2S})(t - I_{1S})] + Z_1[I_{2S} + I_{3S} + I_{1L} + (1 - I_{2S}/I_{2L})(t - I_{1L})]$$

if $Z_0 I_{1S} + Z_1 I_{1L} < t \le Z_0(I_{1S} + I_{2S}) + Z_1(I_{1L} + I_{2L})$, or

$$f = Z_0[I_{3L} + I_{1S} + I_{2S} + (1 - I_{3L}/I_{3S})(t - I_{1S} - I_{2S})] + Z_1[I_{3S} + I_{1L} + I_{2L} + (1 - I_{3S}/I_{3L})(t - I_{1L} - I_{2L})]$$

if $Z_0(I_{1S} + I_{2S}) + Z_1(I_{1L} + I_{2L}) < t \le Z_0(I_{1S} + I_{2S} + I_{3S}) + Z_1(I_{1L} + I_{2L} + I_{3L})$. or

$$f = Z_0(I_{1S} + I_{2S} + I_{3S}) + Z_1(I_{1L} + I_{2L} + I_{3L})$$

if $t > Z_0(I_{1S} + I_{2S} + I_{3S}) + Z_1(I_{1L} + I_{2L} + I_{3L})$

Thus there are six variables that were used to quantify the results of the reciprocal transfers. These variables were estimated by the GAUSS method in the PROC NLIN of the Statistical Analysis Systems Institute version 9.1 (SAS Institute Inc.).

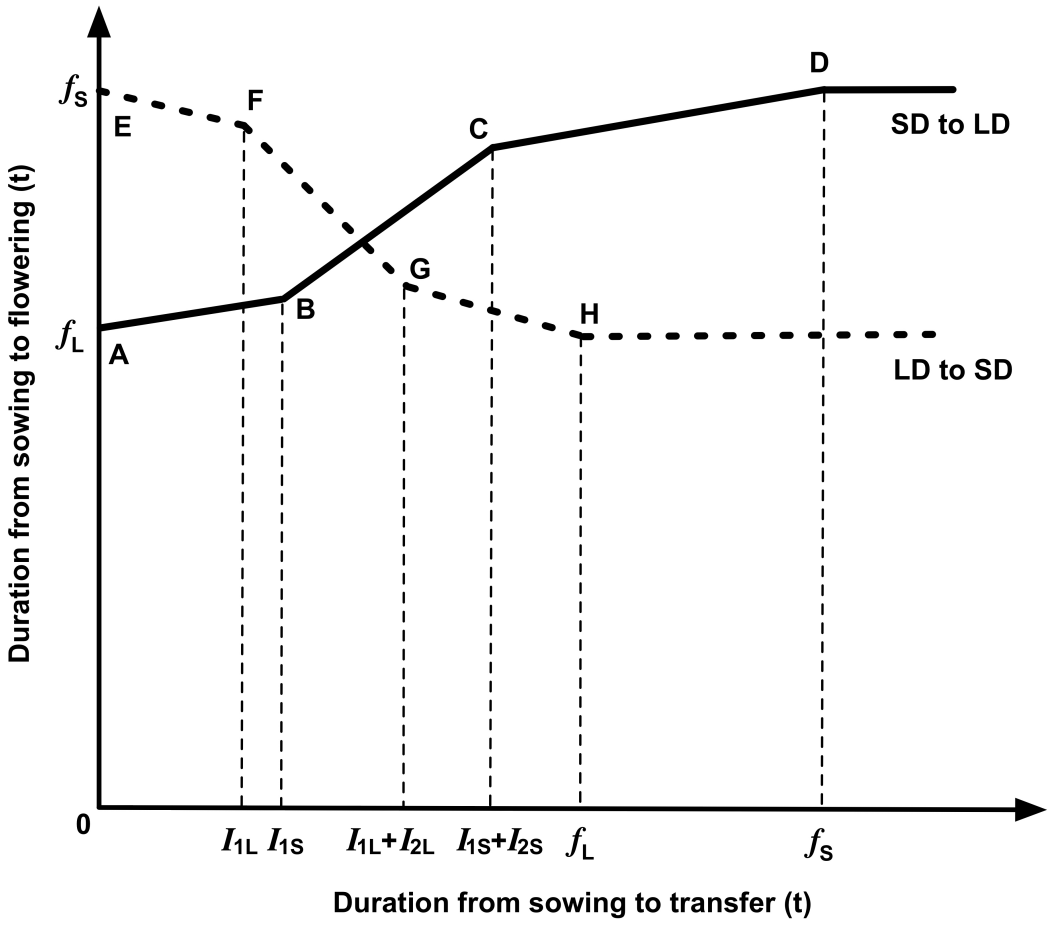


Figure 3.1 Schematic representation of the response of duration from sowing to flowering (*f*) for plants transferred from short day (SD) to long day (LD) conditions (solid lines) or from LD to SD (dashed lines) at various times after sowing (*t*).
Under the assumptions that the period from sowing to flowering consists of three sub-phases, and LD conditions hasten the development of a flax plant more during the second sub-phase than during the other two sub-phases. Linear portions, 'AB', 'BC', and 'CD', represent the three sub-phases under the SD conditions, respectively, and linear portions 'EF', 'FG', and 'GH' represent those under the LD conditions, respectively. In the figure, I_{1S} and I_{1L} denote the length of the first sub-phase under SD and LD conditions, respectively; I_{2S} and I_{2L} denote the length of the second sub-phase under SD and LD conditions, respectively; and I_{3L} and I_{3H} denote the length of the third sub-phase under SD and LD conditions, respectively. Therefore, the duration from sowing to flowering at SD (f_S) can be expressed as $f_S = I_{1S} + I_{2S} + I_{3S}$, and the duration from sowing to flowering at LD (f_L) can be expressed as $f_L = I_{1L} + I_{2L} + I_{3L}$. The dashed arrows downwards indicate the position on the *t*-axis of the points of transition from one sub-phase to another identified from either SD-to-LD or LD-to-SD transfers. The horizontal extension beyond point 'D' for the SD-to-LD transfer and the extension beyond point 'H' for the LD-to-SD transfer take account of the possibility that some plants flower before the set transfer date.

3.5 Results

3.5.1 Determination of the length of different phases

3.5.1.1 Responses to reciprocal transfers

Homogeneity of variances was tested using Levene's test ($p>0.05$) before combining data from two replications of the experiment. Data collected from two rounds of the experiment were combined since the patterns from both rounds were similar to each other. Although for CDC Sorrel, homogeneity of variance of two rounds was significantly different ($p<0.05$).

Variations in flowering time indicate that all cultivars are sensitive to photoperiod changes. Differences between LD and SD controls in all cultivars demonstrate that SD lengthens the vegetative stage of flax plants and as a consequence delays the onset of their reproductive stage. Variations in flowering time indicate that Royal, RE1, RE2 and RE3 are all sensitive to photoperiod changes. Compared with the cultivars, differences between LD and SD controls of RE1, RE2 and RE3 are relatively small, which indicate that these mutant genotypes are less sensitive than the cultivars. Among all mutant genotypes, RE2 has the smallest difference in DTF between LD and SD controls (6.4 days), which implies that RE2 is the least photoperiod sensitive mutant genotype.

Figure 3.1 demonstrates responses to reciprocal transfers between LD and SD conditions of four cultivars (CDC Sorrel, CDC Bethune, Flanders and Prairie Thunder). Control plants of CDC Bethune flowered earliest under both LD and SD conditions among the four cultivars. Its SD control plants flowered 16.9 days later than the LD controls.

Control plants of CDC Sorrel and Flanders had a relatively larger difference in flowering time between LD and SD, which was 28.2 days for both cultivars. For cultivar Prairie Thunder and CDC Bethune, the differences were 24.6 and 17.0 days, respectively.

Figure 3.2 shows that transfers influenced the flowering time of Royal and all RE genotypes. Compared with Royal, the three mutant genotypes RE1, RE2 and RE3 flowered 9.8-16.5 days earlier under LD, and 26.2-36.3 days earlier under SD. RE2 was the earliest under both LD and SD conditions. SD control plants of RE2 flowered only 6.4 days later than LD control plants, and flowering time of RE2 varied within a smaller range (35.0-41.9 days) compared with Royal, RE1 and RE3, indicating that RE2 was least sensitive to photoperiod changes.

The period from seeding to flowering was divided into three phases: BVP (Basic Vegetative Phase), known as the "pre-inductive stage" or "juvenile phase" (Chang et al., 1969), PSP (Photoperiod Sensitive Phase), known as the "inductive stage" and PPP (Post-PSP Phase), known as the "post-inductive stage". During BVP a plant grows vegetatively under the most optimal day length (Chang et al., 1969). For the selected cultivars CDC Sorrel, CDC Bethune, Prairie Thunder and Flanders, although LD conditions accelerated their flowering time, all flax plant individuals flowered under both LD and SD conditions, indicating that these currently grown flax cultivars are facultative LD plant. By applying Yin's model (Yin, 2008), the length of different phases from seeding to flowering under LD and SD conditions was predicted among all flax cultivars and genotypes (Figure 3.4). Duration of the three phases varied among all cultivars and genotypes under LD or SD.

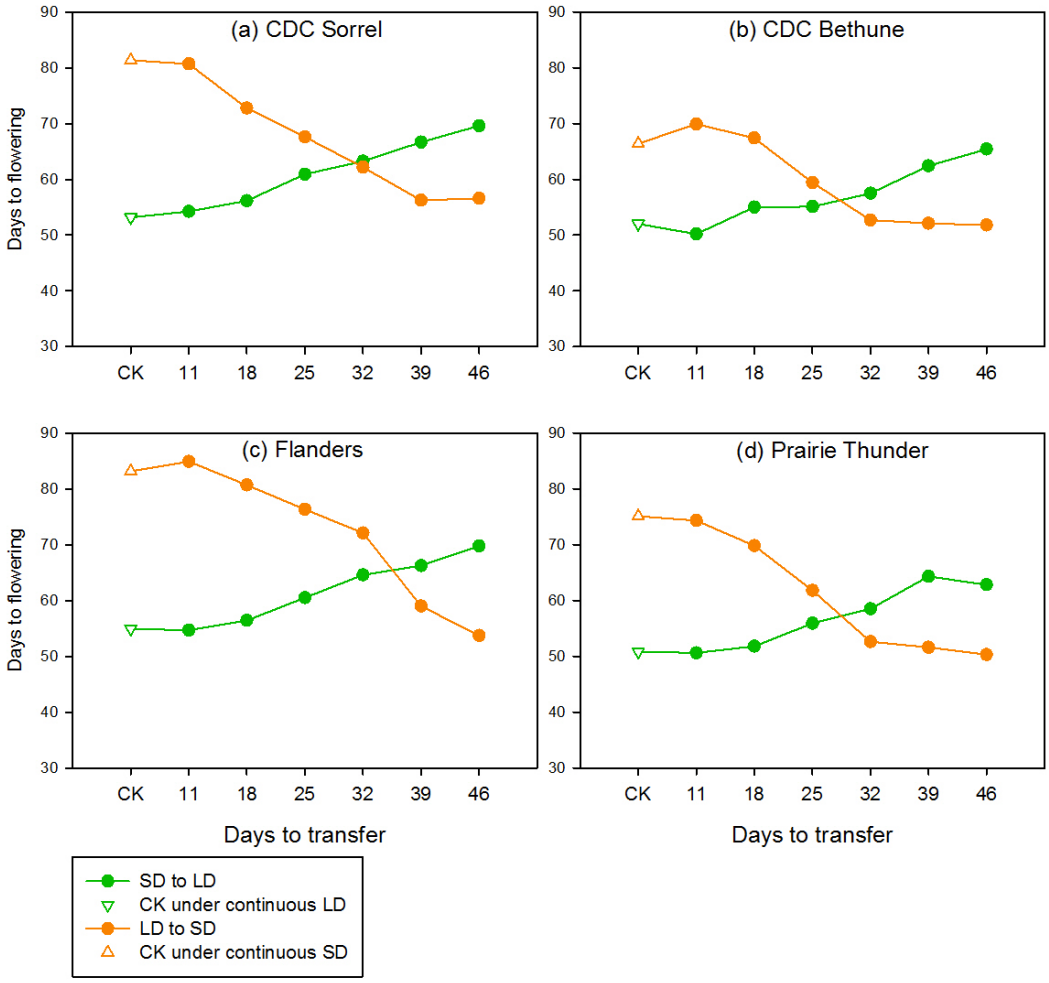


Figure 3.2 DTF versus days to transfer for CDC Sorrel, CDC Bethune, Flanders and Prairie Thunder.
CK plants were not transferred. Numbers 11, 18, 25, 32, 39, 40 were the days the plants stayed in the first chamber before transferring.

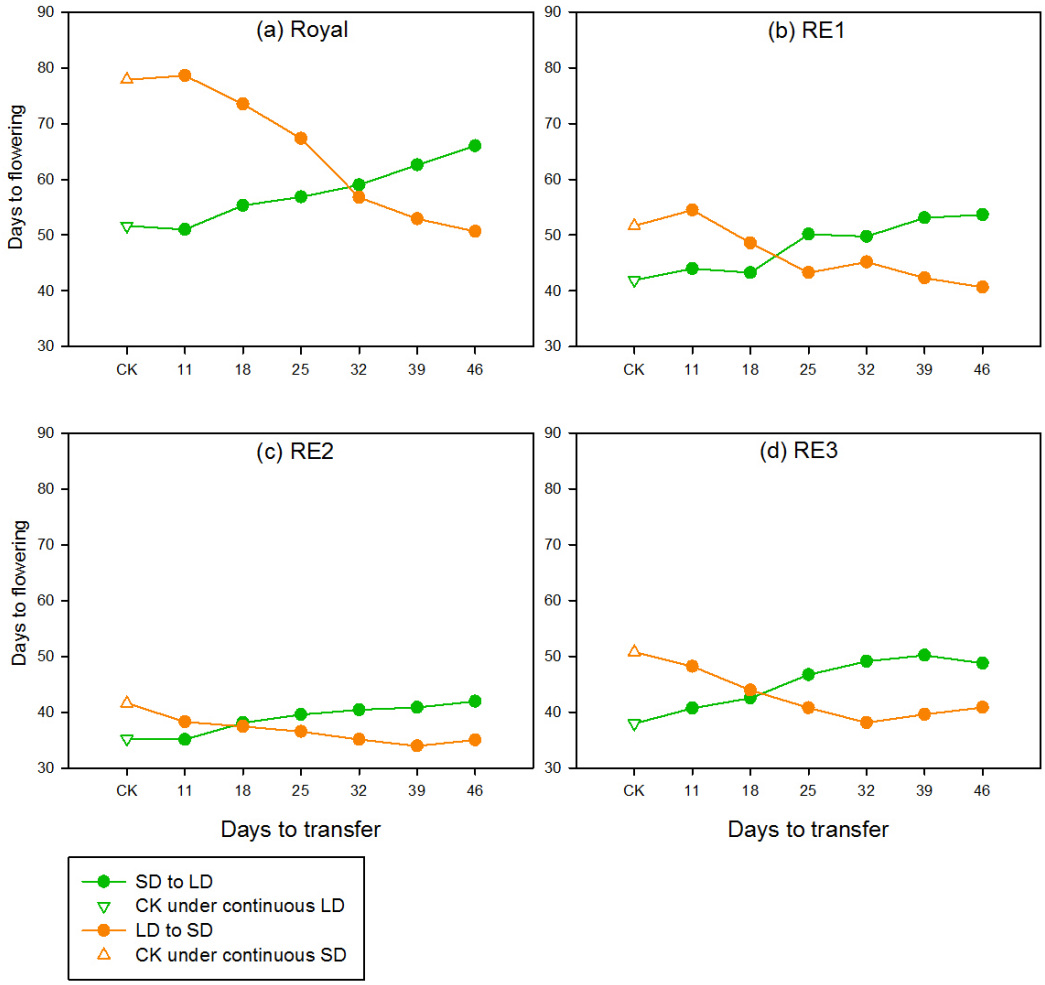


Figure 3.3 DTF versus days to transfer for Royal, RE1, RE2 and RE3.
CK plants were not transferred. Numbers 11, 18, 25, 32, 39, 40 were the days the plants stayed in the first chamber before transferring.

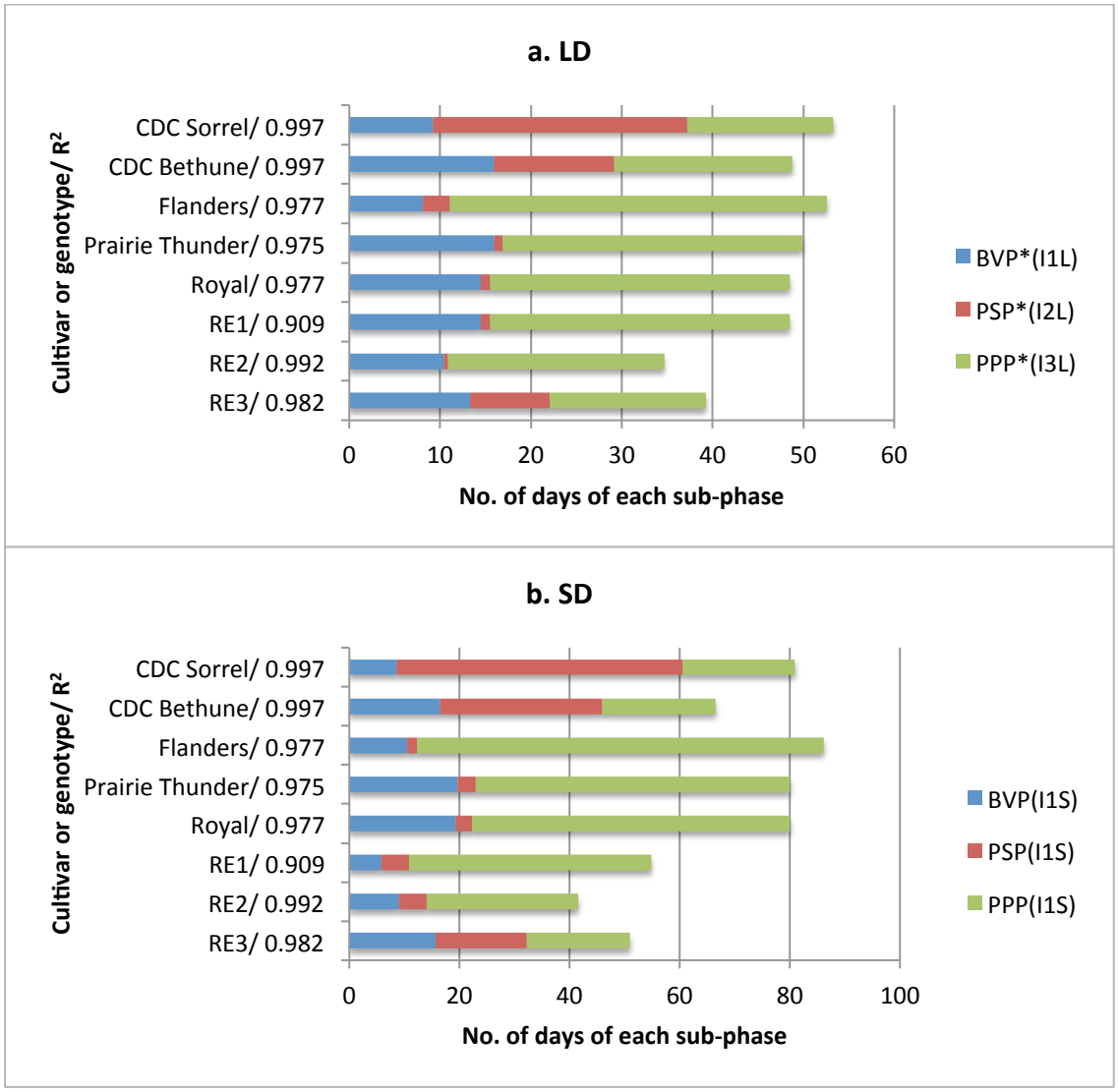


Figure 3.4 Number of days of different sub-phases from sowing to flowering for all selected cultivars/genotypes and model fitness values (R^2).
BVP: Basic vegetative phase; PSP: Photoperiod-sensitive phase; PPP: post photoperiod-sensitive phase.

Under LD, RE2 had the shortest PSP phase among all cultivars and genotypes, which was consistent with what was observed in field tests (see Chapter 5 of this thesis) (Figure 3.2.c). In addition, RE2 had a shorter PPP phase compared with Royal and RE1. Together the shorter PSP and PPP led to early flowering in RE2. This was also consistent with what was observed in the field test (see Chapter 4) that RE2 was the earliest flowering genotype among all cultivars and genotypes.

3.5.2 Tukey Studentized Range (HSD) Test for DTF

3.5.2.1 Comparisons among cultivars

For all five cultivars and three mutant genotypes, the difference between their LD and SD controls were significant.

For CDC Sorrel (Table 3.2), at each transfer, flowering time was later for plants that transferred from LD to SD than those transferred from SD to LD. Plants under continuous LD (transfer 0) flowered much earlier than that under SD (transfer 0'). Whether under LD or SD conditions, changes in time to flowering showed a gradual and stable pattern rather than a sharp increase or decrease between each consecutive transfers. There were no significant differences in DTF among the first transfers compared with others under LD until transfer 5. Meanwhile, under SD, a significant difference first appeared at transfer 3'. This indicated that CDC Sorrel had a more sensitive response to the reduction than increase in day length. The photoperiod sensitivity phase of CDC Sorrel began on approximately the 18^{th} day after seeding, which corresponded to transfers 2 and 2', and ended around the 40^{th} day after seeding, which was approximately at transfers 5 and 5' (Table 3.2).

For CDC Bethune, as is shown in Tukey's test results (Table 3.2), transfers affected DTF of CDC Bethune significantly in the middle stage for plants transferred from LD to SD (transfer 3'). CDC Bethune plants that were transferred from LD to SD flowered later compared with those transferred from SD to LD plants. The same pattern was observed with CDC Sorrel, which indicated that plants under LD flowered significantly earlier than under SD. Furthermore, a significant difference did not show until the fifth transfer under LD. A significant difference appeared at the third transfer under SD.

Flanders flowered significantly earlier under LD than under SD condition (Table 3.2). These results indicated that Flanders was more sensitive to photoperiod changes since the flowering time of plants transferred at the same time had a larger difference in days to flowering between SD and LD conditions than that of CDC Sorrel and CDC Bethune. When comparing the first transfer with the later transfers under LD, a significant difference appeared at transfer 4, which was one week earlier than that observed in both CDC Sorrel and CDC Bethune. Plants transferred 39 days after seeding (transfer 5') (LD to SD) flowered significantly earlier than those of transfer 4' (LD to SD).

For Prairie Thunder, a significant difference of flowering time appeared at transfer 4 compared with transfer 1 under LD (Table 3.2). There are also significant changes between transfer 2', 3' and 4' which were transferred at 18, 25, 32 days after seeding respectively from LD to SD.

Table 3.1 Tukey Studentized Range (HSD) Test for DTF for cultivars and genotypes.

	CDC Sorrel		**CDC Bethune**		**Flanders**		**Prairie Thunder**	
Transfer	Tukey Grouping	Mean	Tukey Grouping	Mean	Tukey Grouping	Mean	Tukey Grouping	Mean
0 (LD CK)	E	53.2	DE	52.0	H	54.9	F	50.8
1	E	54.3	E	50.2	H	54.7	F	50.6
2	DE	56.2	CDE	55.0	GH	56.4	F	51.8
3	CDE	60.9	CDE	55.1	FGH	60.5	EF	55.9
4	BCDE	63.3	CDE	57.5	EFG	64.6	CDE	58.5
5	BCD	66.7	ABC	62.4	EF	66.3	BC	64.3
6	BC	69.6	AB	65.4	CDE	69.8	C	62.8
0' (SD CK)	A	81.3	AB	66.4	AB	83.2	A	75.1
1'	A	80.7	A	69.9	A	87.8	A	74.3
2'	AB	72.8	A	67.4	ABC	80.7	AB	69.8
3'	BC	67.6	BCD	59.4	BCD	76.3	CD	61.8
4'	BCDE	62.2	DE	52.7	CDE	72.1	EF	52.6
5'	DE	56.3	DE	52.1	FGH	59.0	F	51.6
6'	DE	56.6	DE	51.8	H	53.8	F	50.3
	Royal		**RE1**		**RE2**		**RE3**	
Transfer	Tukey Grouping	Mean	Tukey Grouping	Mean	Tukey Grouping	Mean	Tukey Grouping	Mean
0’ (LD CK)	FG	51.7	DE	41.9	EFG	35.2	F	37.9
1	G	51.0	CDE	44.0	EFG	35.1	DEF	40.7
2	EFG	55.3	CDE	43.3	BCDE	38.1	CDE	42.5
3	EFG	56.8	ABC	50.2	ABCD	39.5	ABC	46.7
4	DEF	59.0	ABC	49.8	ABC	40.4	A	49.1
5	CDE	62.6	A	53.1	AB	40.8	A	50.2
6	CD	66.0	A	53.7	A	41.9	A	48.8
0' (SD CK)	A	77.8	AB	51.7	A	41.6	A	50.8
1'	A	78.6	A	54.5	BCD	38.3	AB	48.2
2'	AB	73.5	ABCD	48.6	CDEF	37.4	BCD	43.9
3'	BC	67.3	CDE	43.3	DEFG	36.5	DEF	40.8
4'	EFG	56.8	BCDE	45.2	EFG	35.1	EF	38.1
5'	FG	52.9	DE	42.3	G	33.9	DEF	39.6
6'	G	50.7	E	40.7	FG	35.0	DEF	40.8

This table illustrates the effect on time to flowering for all five cultivars and three RE genotypes grown under LD (Transfer 0, CK) or SD (Transfer 0’, CK), and after transferring from SD to LD (1-6) or LD to SD (1’-6’) environments. Comparisons were conducted within each genotype. Different letters show the significant difference (p=0.01) in flowering time according to Tukey’s Studentized Range (HSD) Test.

3.5.2.2 Comparisons among Royal and its mutant genotypes

For Royal, DTF for transfers 1 and 4 were significantly different under LD (Table 3.2), which indicated that Royal had an earlier sensitive phase compared with CDC Sorrel and CDC Bethune. Under SD, transfer 3' flowered significantly earlier than transfer 1', and transfer 5' flowered significantly earlier than transfer 3' (Table 3.2).

RE1 (Table 3.2) was less sensitive to changes in day length and there was less significant variation in time to flowering due to the responses to LD to SD or SD to LD transfers compared with Royal (the background cultivar) and other cultivars CDC Sorrel, CDC Bethune, Flanders and Prairie Thunder.

For RE2, the results (Table 3.2) showed that DTF under different transfers were similar to each other. RE2 was much less photoperiod sensitive than Royal, Prairie Thunder, Flanders, CDC Sorrel and CDC Bethune.

For RE3, as was shown in Tukey's tests results (Table 3.2), RE3 had a very early photoperiod sensitivity stage. There was no significant difference between first transfers and controls. In addition, when comparing the first transfers with later transfers, under LD, a significant difference appeared at the third transfers. In the later stage under both LD and SD, flowering time appeared to be relatively uniform.

3.5.3 Correlation among traits

In CDC Sorrel, all traits measured (DTF, NON, HT and HTFB) were significantly related (Table 3.3). As was previously reported, NON can be a useful indicator of time to flowering in flax (Fieldes and Harvey, 2004). For CDC Bethune, all four traits were significantly related ($p < 0.0001$) although the R^2 values were moderate for HT, DTF and NON. All four traits were significantly and highly correlated in Flanders. In Prairie

Thunder, all four traits were highly and significantly correlated as was shown in other cultivars, CDC Bethune, CDC Sorrel and Flanders.

For Royal and its mutant genotypes (Table 3.3), all four traits were highly and significantly correlated in Royal. In RE1, correlation analysis revealed that HT and DTF, HT and NON were not significantly correlated. Other traits in RE1 such as DTF and NON, HT and HTFB were significantly correlated. In RE2, correlation analysis suggested that four traits DTF, NON, HT and HTFB are all significantly correlated with each other. And in RE3 all traits listed were significantly correlated with each other.

Table 3.2 Correlation of traits for all cultivars and genotypes.

Cultivar/Genotype	Trait	DTF[1]	NON[2]	HT[3]	HTFB[4]
CDC Sorrel	DTF	1.000			
	NON	0.822***	1.000		
	HT	0.605***	0.633***	1.000	
	HTFB	0.629***	0.667***	0.818***	1.000
CDC Bethune	DTF	1.000			
	NON	0.864***	1.000		
	HT	0.347***	0.388***	1.000	
	HTFB	0.624***	0.640***	0.671***	1.000
Flanders	DTF	1.000			
	NON	0.889***	1.000		
	HT	0.556***	0.702***	1.000	
	HTFB	0.743***	0.806***	0.807***	1.000
Prairie Thunder	DTF	1.000			
	NON	0.868***	1.000		
	HT	0.481***	0.754***	1.000	
	HTFB	0.624***	0.844***	0.929***	1.000
Royal	DTF	1.000			
	NON	0.883***	1.000		
	HT	-0.005	0.117	1.000	
	HTFB	0.648***	0.690***	0.162*	1.000
RE1	DTF	1.000			
	NON	0.740***	1.000		
	HT	0.150	0.164*	1.000	
	HTFB	0.568***	0.673***	0.409***	1.000
RE2	DTF	1.000			
	NON	0.612***	1.000		
	HT	0.462***	0.332***	1.000	
	HTFB	0.500***	0.653***	0.475***	1.000
RE3	DTF	1.000			
	NON	0.743***	1.000		
	HT	0.300***	0.158*	1.000	
	HTFB	0.774***	0.732***	0.384***	1.000

[1]DTF: Days to flowering;
[2]NON: Number of nodes;
[3]HT: Height;
[4]HTFB: Height to the first branch.
The correlation tests were conducted within each cultivar/genotype. In the table, *** indicates significant level at $p< 0.001$, ** for $p< 0.01$ and * for $p< 0.05$.

3.6 Discussion

Flax is a LD plant and DTF is impacted by different photoperiod (LD versus SD), these results are supported by other studies (Singh, 2010; Zhang, 2013). In all cultivars and genotypes, control plants grown under LD has significantly shorter DTF than that of a SD, which meant that flowering is delayed under SD and accelerated under LD. Moreover, transfers from LD to SD influences DTF for all cultivars and genotypes, and *vice versa*.

The response to photoperiod changes varies among cultivars and genotypes. For instance, CDC Sorrel, CDC Bethune, Prairie Thunder and Royal have a later photoperiod sensitive phase compared with Flanders, RE1, RE2 and RE3. The magnitude of the influence of photoperiod on DTF differs among the cultivars and genotypes studied. For example, RE2 is the least photoperiod sensitive genotype followed by RE3, RE1, Flanders, CDC Bethune, Royal, Prairie Thunder and CDC Sorrel.

Traits correlated with DTF in this study include NON, HT and HTFB. Plant HT and HTFB reduced in plants grown under LD as compared with SD, suggesting that growth under LD shortens the vegetative stage, reducing the time to flowering and extending the reproductive phase in flax cultivars and genotypes. In early flowering genotypes, the vegetative stage is shortened compared with their respective control genotypes as found by Fieldes and Harvey (2004). Early flowering limits vegetative growth, enables reproductive growth to occur before terminal stress, and usually correlates with early maturity (Serraj et al., 2004). Early flowering in chickpea (*Cicer arietinum*) leads to earlier maturity by 10 days (Anbessa et al., 2007).

The four traits (DTF, NON, HT and HTFB) are significantly correlated with each other in CDC Sorrel, CDC Bethune, Prairie Thunder and Flanders, whereas inin Royal an RE1 these traits were not significantly correlated In Royal and RE1/2/3, NON is significantly correlated with DTF. These results are supported by Fieldes and Harvey (2004), whose work suggests that node/leaf number could be a useful indicator in predicting DTF in flax. Thus, both NON and HTFB can be used as indications for predicting flowering time.

3.7 Future work

The variation found in photoperiod responses between Royal and its early flowering derivative genotypes (RE1, RE2 and RE3) may be related to their methylation pattern difference. In the future this study can be extended to examine the methylation and expression patterns of different genes that control flowering time in the cultivar Royal and its early flowering derivative genotypes.

CHAPTER 4

4. *ELF4* gene expression pattern in flax

4.1 Abstract

EARLY FLOWERING 4 (ELF4) was first discovered in *Arabidopsis*. It is an important gene in perceiving photoperiod and coordinating circadian clock in plants. Three orthologues of *ELF4* were present in the flax draft genome. The expression pattern of these three *ELF4* orthologues was examined in three flax cultivars (CDC Sorrel, CDC Bethune and Royal) and a 5-azaC treated early flowering mutant of the cultivar Royal (RE2) using RT-qPCR. CDC Sorrel and CDC Bethune had similar expression patterns, and Royal and RE2 had similar expression patterns. However, the expression pattern of *ELF4* in CDC cultivars was different from Royal and RE2. It is unlikely that demethylation occurred at the *ELF4* locus as RE2, a demethylated early flowering derivative genotype of Royal, flowers much earlier than its original germplasm, Royal, although the expression of *ELF4* was similar.

4.2 Introduction

Regulation of the circadian clock and flowering time has been studied in a number of plant species, and they are understood most thoroughly in Arabidopsis (Doyle et al., 2002; Kikis et al., 2005; Hopkins, 2006; McClung, 2006b; Staiger et al., 2013; Endo et al., 2014). The circadian clock consists of three major components: input pathways, a central oscillator, and output pathways(Hopkins, 2006; McClung, 2006a). The central oscillator is the key to a endogenous circadian clock, which expresses a self-sustaining rhythm under constant light or dark conditions (i.e. free-running conditions, constant light or dark for 24 h) (Hopkins, 2006). Regulation of circadian clock and flowering is through the expression of interacting genes involved in both pathways. Examples of these genes

include the flowering initiation genes *GIGANTEA (GI), EARLY FLOWERING3 (ELF3), ELF4,* and *LUX ARRHYTHMO (LUX),* all of which play a role in regulating the expression of the circadian clock genes *CCA1* and *LHY* (Figure 2.4) (Doyle et al., 2002; McClung, 2006b; Staiger et al., 2013; Endo et al., 2014). One important regulator of the circadian clock is *EARLY FLOWERING 4 (ELF4).* This gene is mainly expressed during the night and is involved in perceiving photoperiod and coordinating the circadian clock in Arabidopsis (Doyle et al., 2002). In Arabidopsis the expression of *ELF4* delays flowering. In this feedback loop, *ELF4* expression promotes the translation and transcription of *CCA1* and *LHY*, which in turn, inhibits the expression of *ELF4*. It was found that the expression of *ELF4* is important to the central oscillator in Arabidopsis (Kikis et al., 2005; Wang et al., 2011). It was also confirmed that *ELF4* is closely related to the *CCA/LHY-TOC1* feedback loop, as *ELF4* is necessary for *CCA1* and *TOC1* expression under free-running condition (McWatters et al., 2007). Overexpression of *ELF4* in plants results in a longer circadian period and delays flowering time (McWatters et al., 2007). *ELF4* mutants (*elf4*) had weaker rhythmicity under free-running condition and exhibited early flowering under a non-inductive photoperiod (Doyle et al., 2002; Kikis et al., 2005; McWatters et al., 2007). This indicated that under constant light/ dark environment, rhythmicity in *elf4* mutants was disrupted, which vacillated around a 24 h cycle.

Reverse Transcriptase quantitative PCR (RT-qPCR) was used to investigate the expression pattern of the flowering-time control gene *ELF4* in CDC Sorrel, CDC Bethune, Royal and RE2. CDC Bethune and CDC Sorrel are two widely grown Canadian flax cultivars that together account for 2/3 of total flax acreage in Canada. CDC Sorrel is a

relatively new cultivar, which has good resistance to shattering, lodging and capsule loss. CDC Bethune is a check cultivar (yield standard) utilized in the linseed flax co-operative trials. Royal is an old flax cultivar, which was developed by plant breeders in the Department of Plant Sciences at University of Saskatchewan and distributed in 1939. It became a popular cultivar in the 1940s (Canada Department of Agriculture and McGregor, 1953). Royal is no longer widely used as newer flax cultivars with better agronomic characteristics and better climatic adaption have been released. RE2 is one of the 5-azaC treated early flowering derivative genotypes (Fieldes, 1994).

This study was conducted to examine expression patterns of *ELF4* orthologues in CDC Bethune, CDC Sorrel, Royal and RE2 shoot tips using RT-qPCR.

4.3 Materials and methods

4.3.1 Plant material and tissue collection

CDC Sorrel, CDC Bethune, Royal and RE2 were grown for tissue collection in controlled environment chambers at the University of Saskatchewan (22°C/18 h during the day with 350-400 μmol $m^{-2}s^{-1}$ light intensity and 17°C /6 h during the night). Shoot tips (~ 1 mm) from all cultivars and genotypes were collected in pre-cooled 1.5 ml Eppendorf tubes on ice every four days around 10:00 am (approximately four hours after the start of the day period), starting at 14 days after seeding. For CDC Sorrel and CDC Bethune, nine time points were obtained in total, with three plants collected separately for each cultivar/genotype at each time point. However, Royal and RE2 flowered earlier than the other cultivars and lines and thus fewer time points were collected than CDC Sorrel and CDC Bethune. Eight time points were obtained for Royal and six time points were obtained for RE2, respectively.

4.3.2 RT-qPCR

Three orthologues of *ELF4* were identified using Basic Local Alignment Search Tool (BLAST) (Altschul et al., 1990) in the draft flax genome (available at http://www.phytozome.net/cgi-bin/gbrowse/flax/), using the Arabidopsis *ELF4* sequence (NCBI Reference Sequence: NM_129566.2). The three flax genes (Lus10018237, Lus10028288, Lus10040667) with the greatest homology (over 70% of nucleotide identity in the open reading frame) to the Arabidopsis *ELF4* were selected for this study. Taqman primers and probes were designed to assay gene expression levels of the *ELF4* orthologues (Figure 4.1 and Table 4.1). One primer set and three probes were designed for the candidate genes (Table 4.1). The housekeeping gene *GAPDH* was selected as reference gene in this study not only because it is a commonly used reference gene in plants, but also because its expression is stable among many candidate housekeeping genes in flax (Huis et al., 2010).

Each of the *ELF4* orthologues being studied was amplified and cloned into pBluescript from genomic DNA to act as positive controls during the PCR. PCR was conducted using genomic DNA as the template and the common *ELF4* primers (Table 4.1) as there are no introns in the target sequence. The PCR products were examined by agarose gel electrophoresis. If the products were bright and clear on the gel, they were gel purified, treated with T4 polynucleotide kinase and ligated into EcoRV cut pBluescript II. An aliquot of the ligation mixture was used to transform *E. coli* DH5a cells. The transformation mixture was plated onto LB+amp+Xgal+IPTG plates for blue and white screening to identify colonies containing plasmids with an insert. White colonies were

Table 4.1 Primers and probes designed for *ELF4*

Primers and probes of *ELF4*	Sequence (5' to 3')	Length (nt)
Primer (Forward)	GASCTGATCSAKCAGGTKAACG	22
Primer (Reverse)	ATSGWCRWGAYYTTGGAGATGTTC	24
Probe 8 (Lus10018237)	CAGTCGAAGATCCCGGCCA	19
Probe 9 (Lus10028288)	CAACATGGCGAAGAACGTGCC	21
Probe 10 (Lus10040667)	TGAGCAGGAACGTCTCCCTGATAAACGAC	29

IUPAC nucleotide code: S for G or C, K for G or T, W for A or T, T for C or T.
(http://www.bioinformatics.org/sms/iupac.html)

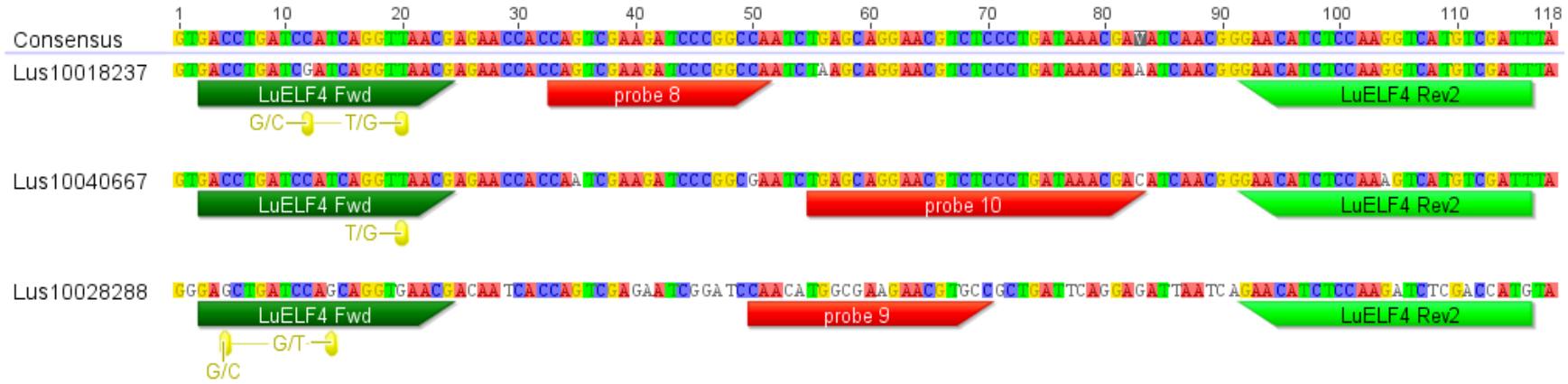


Figure 4.1 Alignment showing the sequences of three orthologues of *ELF4*, forward/reverse primer, and three probes. The three flax genes with the greatest homology to the *Arabidopsis ELF4* were identified and their sequences aligned, as shown. The forward and reverse primers are shown with the right and left pointing arrows, respectively, while the three probes are indicated in red. Yellow symbols indicate mismatch in the primers compared to the gene sequence.

used to inoculate LB+amp broth and the plasmid extracted using a Qiagen plasmid mini kit.

Plasmid was digested with restriction enzymes to release the fragment from the vector backbone. This digest was examined on a gel to determine if a fragment of the expected size was present or not. Plasmids containing fragments of the expected size were sequenced at the DNA Sequencing Facility, NRC-Saskatoon.

RNA extraction was conducted right after tissue collection using a QIAGEN RNeasy Plant Mini Kit. Genomic DNA was removed using gDNA Wipeout from the Omniscript Reverse Transcriptase kit (Qiagen).

A two-step RT-qPCR procedure was used to determine *ELF4* orthologue expression levels. Reverse-transcription was performed on 1μg total RNA using random hexamers as primers and Omniscript Reverse Transcriptase (Qiagen). Quantitative PCR was performed on 1.5 μl cDNA as follows: a 15 μl total volume, 2 μl cDNA template, 7.5 μl 2 x SsoFast qPCR supermix, 300 nM primer (forward), 300 nM primer (reverse), 200 nM probe 8, 100 nM probe 9, 100 nM probe 10 and 4 μl H_2O. The following amplification program was used in a BioRad CFX384 machine: 95°C, 2 min; 95°C, 10 sec; 54°C, 15 sec, followed by a plate read for 46 cycles. Amplification of the reference gene, GAPDH was performed on the same plate using the same cDNA, but in different wells as there was interference between the qPCRs.

4.3.3 Data analyses

RT-qPCR data were analyzed using the Δ*Ct* method (Livak and Schmittgen, 2001). As there was no calibration in this experiment, a comparative quantification algorithm (Δ*Ct*) method was used for data analysis. In each cultivar/genotype, Δ*Ct* was obtained by

subtracting the C_t value of reference gene ($C_{t\ norm}$) from the *Ct* value of the samples ($C_{t\ sample}$).

$\Delta Ct = C_{t\ sample} - C_{t\ norm;}$ (a)

Then, the fold difference was calculated as an indication of the relative expression level of each orthologue.

Fold difference = $2^{-\Delta Ct}$. (b)

As a reference gene, *GAPDH* was used as it is usually expressed stably. This means that $C_{t\ norm}$ is a relatively stable value in this equation (a). Thus, a larger ΔCt value stands for a larger $C_{t\ sample}$ value, which means that sample genes had a lower concentration relative to the reference gene, *GAPDH*. The fold difference in equation (b) is used to compare expression patterns of different orthologues of the *ELF4* gene. For the expression of an orthologue, x-fold difference means there is x time(s) as much expression as the reference gene, where x can be any positive number.

Fold differences from each time point, cultivar/genotype and replicate were calculated individually. The mean and standard deviation were calculated for each time point and cultivar using each replicate as a measure (n=3). Multiple comparisons among time points of each cultivar/genotype were performed using Tukey's Studentized Range (HSD) test at $p<0.05$ confidence level in SAS 9.0 (SAS Institute Inc., Cary, NC, USA.).

4.4 Results

On average, CDC Sorrel and CDC Bethune flowered 50 and 48 days after seeding, respectively, while Royal and RE2 flowered 42 and 38 days after seeding, respectively. Since RNA concentration was too low at the fourth time point in both Royal and RE2,

there was no cDNA generated to obtain results in the RT-qPCR. C_t values of the reference gene *GAPDH* are shown in Figure 4.2.

Overall, within each cultivar/genotype the level of *ELF4* orthologue expression was similar across the time course of the experiment, except for a single, significantly different time point within each cultivar/genotype. Among all four cultivars and genotypes, CDC Sorrel and CDC Bethune exhibited similar expression patterns, while Royal and RE2 exhibited similar expression patterns.

4.4.1 Probe 8

A comparison of *ELF4* orthologue expression in CDC Sorrel and CDC Bethune showed similar expression patterns (Figure 4.3). Overall, the expression was stable across the time course except for the peaks in Royal and RE2.

For CDC Sorrel, *ELF4* expression increased from the 14th day to the 18th day. At the 22nd day the fold difference increased significantly to 1.37×10^{-3}, followed by a significant decrease on the 26th day. Starting from then, the expression remained stable and no significant difference was observed.

For CDC Bethune, *ELF4* expression was higher at all the time points when compared with CDC Sorrel. The expression level on the 22nd day after seeding (fold increase 2.64×10-3) was twice as much as the 14th day after seeding. Tukey's Studentized Range (HSD) test ($p<0.05$) results showed that the expression level at the third time point was significantly higher than the first two time points. On the 26th day, the expression level was significantly lower than the 22nd day and remained stable in the following time points.

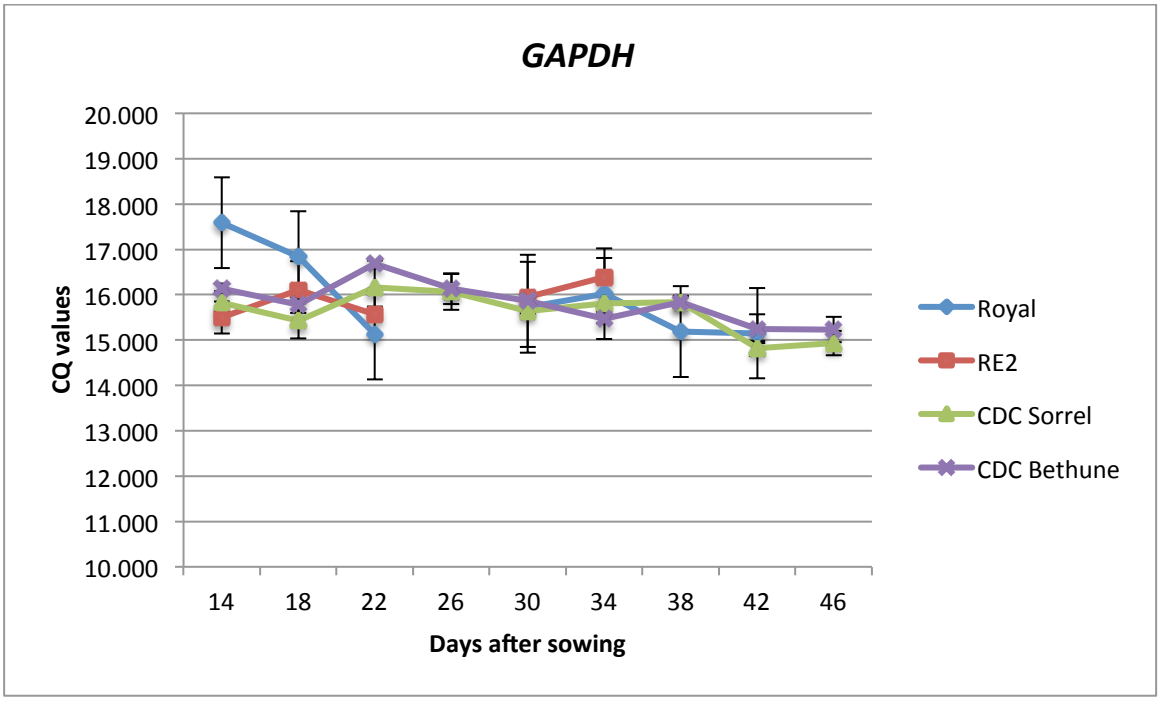


Figure 4.2 C_t values of the reference gene *GAPDH* at different sampling time points. As reference gene, *GAPDH* in all cultivars and genotypes had relatively similar expression pattern. The bars on each point indicate the standard deviation (SD) of C_t value for each time point.

Compared with CDC Sorrel and CDC Bethune, Royal and RE2 flowered much earlier. Royal and RE2 also had a different pattern of *ELF4* expression (Figure 4.3). From the 14^{th} to the 30^{th} day after seeding there was no significant difference in the expression (the 26th day was missing). The only significant change appeared at the 34^{th} day for both Royal and RE2, at which point the expression levels were four times higher than the 14^{th} day. In Royal expression significantly decreased to a lower level on the 30th day.

4.4.2 Probe 9

In CDC Sorrel and CDC Bethune (Figure 4.4), *ELF4* orthologue expression pattern appeared to be less variable compared with those observed with probe 8. In CDC Sorrel, the expression pattern was stable from seeding to the 30^{th} day. *ELF4* orthologue expression didn't significantly change until a significant decrease on the 34^{th} day. On the 38^{th} day it increased significantly followed by another significant decrease on the 42^{nd} day. On the 46^{th} day it significantly increased again.

In CDC Bethune, the expression level was higher at all the time points compared with CDC Sorrel, consistent with the probe 8 results. The fold increase on the 22^{nd} day (8.74×10^{-4}) was significantly higher than both the 14^{th} day (5.00×10^{-4}) and the 18^{th} day (4.19×10^{-4}). On the 34^{th} day, the expression level was significantly lower than the 26^{th} day, which remained stable in the later time points.

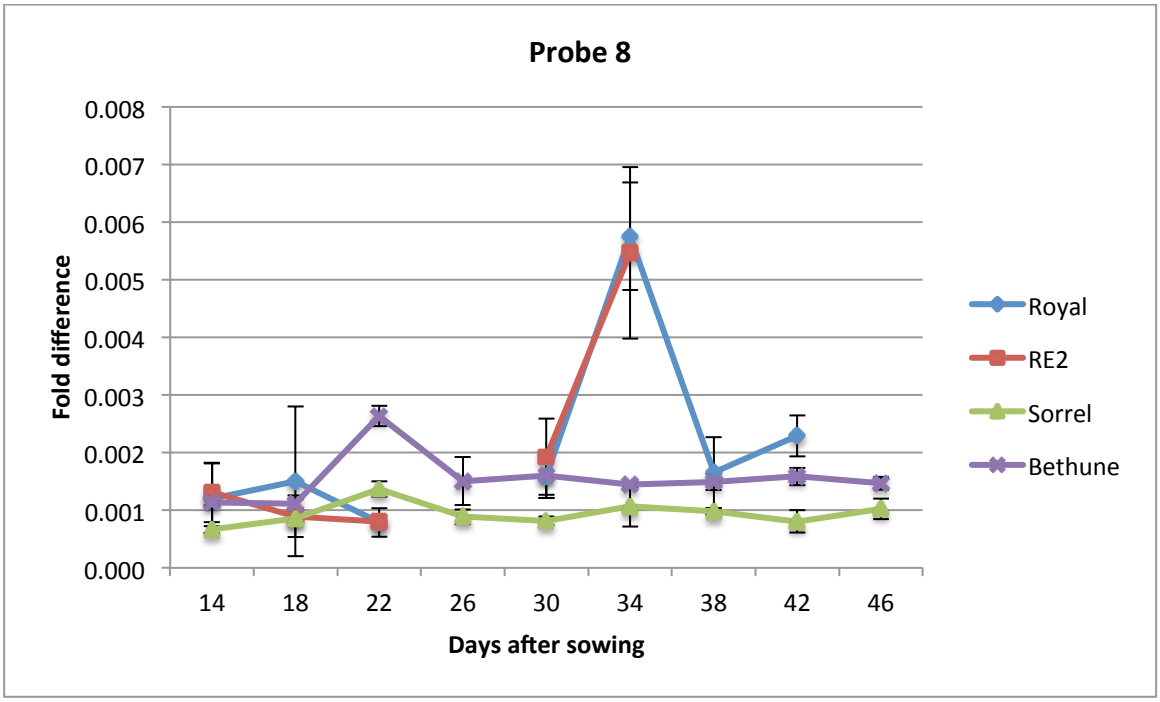


Figure 4.3 Comparison of *ELF4* expression patterns in all cultivars and genotypes with probe 8.
Shown as a comparison of fold differences of ΔC_t values. On average CDC Sorrel, CDC Bethune, Royal and RE2 flowered 50, 48, 42 and 38 days after seeding, respectively. The bars on each point show the standard deviation for each time point.

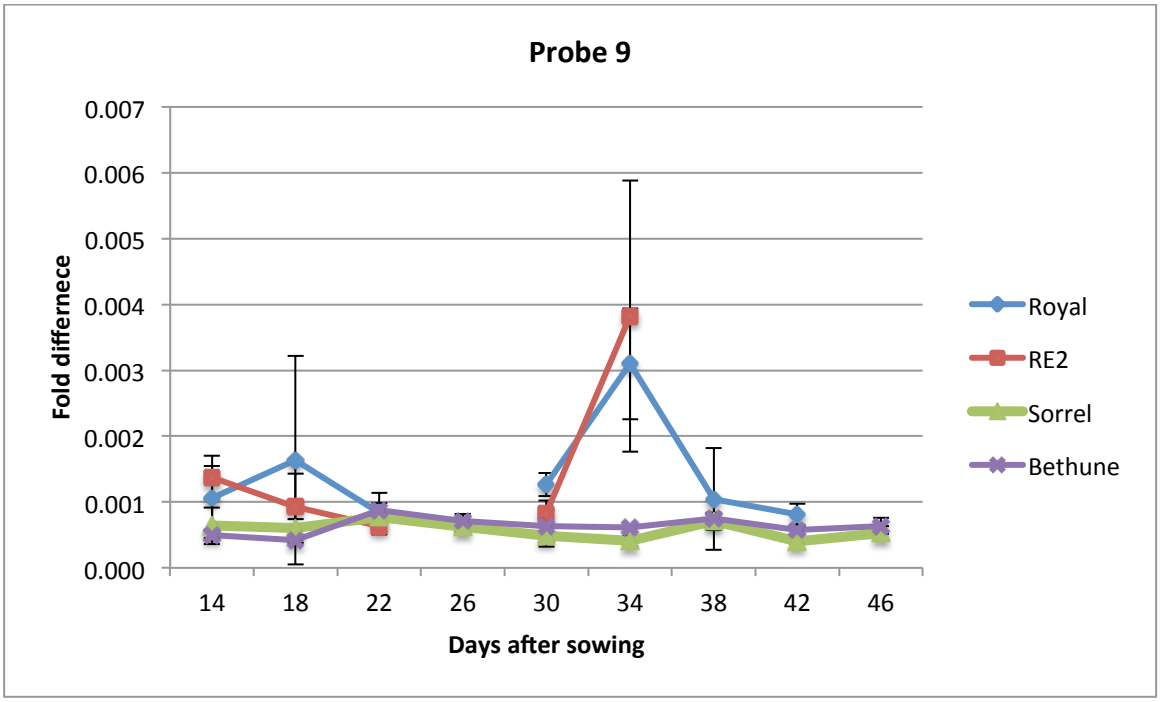


Figure 4.4 Comparison of *ELF4* expression patterns in all cultivars and genotypes with probe 9.
Shown as a comparison of fold differences of ΔC_t values. On average CDC Sorrel, CDC Bethune, Royal and RE2 flowered 50, 48, 42 and 38 days after seeding, respectively. The bars on each point show the standard deviation for each time point.

Royal and RE2 also had a different trend for the expression of the probe 9 in *ELF4* orthologue compared to the probe 8 results. There was no significant difference in the expression (the 26th day time point was missing) from the 14th to the 30th day. The only significant change appeared on the 34th day for both Royal and RE2. The fold difference in Royal and RE2 increased to 3.0 and 4.4 times, respectively, on the 34th day compared with that of the 14th day. In Royal it significantly decreased to a lower level and remained stable for the rest of the time course.

4.4.3 Probe 10

A comparison of CDC Sorrel and CDC Bethune (Figure 4.5) showed peaks in *ELF4* orthologue expression in both cultivars on the 22nd day after seeding. From seeding to the 18th day after seeding, there were no significant changes in *ELF4* orthologue expression in either CDC Sorrel or CDC Bethune.

For CDC Sorrel, *ELF4* expression increased significantly from the 14th day (1.38×10^{-3}) to the 18th day (1.68×10^{-3}) and the 22nd day (2.44×10^{-3}). This increase in expression was followed by a significant decrease on the 26th day (1.74×10^{-3}) back to a stable level of expression.

For CDC Bethune, the expression level was higher at all the time points compared to CDC Sorrel. The expression level at the 3rd time point (fold increase 4.78×10^{-3}) was significantly higher than the first (2.08×10^{-3}) and the second time points (2.02×10^{-3}). On the 26th day, the expression level dropped significantly to 2.73×10^{-3} and remained stable in the later time points.

Again Royal and RE2 had a different trend in *ELF4* expression compared with CDC cultivars. From the 14th to 39th day after seeding, there was no significant difference in

the expression (the 26^{th} day was missing). The only significant change also appeared on the 34^{th} day after seeding for both Royal and RE2, at which time point the fold increase was 4.4 times and 4 times higher than the first time point for Royal and RE2, respectively. Then in Royal it significantly decreased to 2.77×10^{-3} and remained stable afterwards.

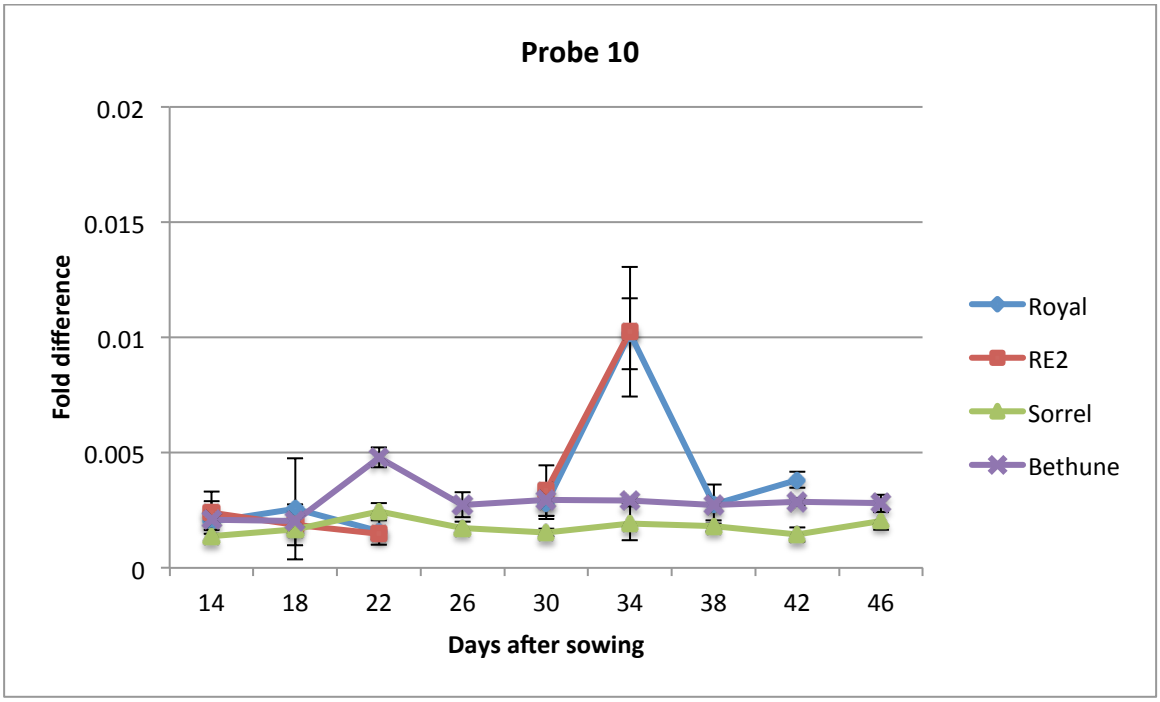


Figure 4.5 Comparison of *ELF4* expression patterns in all cultivars and genotypes with probe 10.
Shown as a comparison of fold differences of ΔC_t values. On average CDC Sorrel, CDC Bethune, Royal and RE2 flowered 50, 48, 42 and 38 days after seeding, respectively. The bars on each point stand for the standard deviation of C_t value of each cultivar/genotype.

4.5 Discussion

Flowering time varied among all cultivars and lines. CDC Sorrel and CDC Bethune flowered 45 and 50 days after seeding on average, while Royal and RE2 showed earlier flowering time (42 and 38 days respectively on average). These results are similar to previous observations (see Chapter 3 of this thesis), and reflect the pattern of flowering times observed in field tests.

In this study, the fold differences for all *ELF4* orthologues were at very low level, indicating that gene expression was low relative to GAPDH. It was reported that *ELF4* participated in the response to red light and was mainly expressed at night (McWatters et al., 2007). More specifically, under a 12 h/12 h condition, its peak expression occurred approximately 4 h after the dark cycle started (Staiger et al., 2013). However, this was not taken into consideration when the experiment was conducted. Higher expression levels would be expected if tissue collection was done at night. Doing so would give a more accurate estimation of the expression pattern of *ELF4* in the genotypes studied. In addition, gene expression pattern comparisons are often made between wild type plants and mutant plants where the $\Delta\Delta Ct$ method is often used in the analysis (McWatters et al., 2007; Cheon et al., 2011). In the $\Delta\Delta Ct$ methods comparisons are made using relative fold differences. However, in this study ΔCt method was used in the data analysis since comparisons of gene expression pattern were made within the same cultivar. Because of the stability in the expression of *GAPDH*, results from this study can still be referred to for future research.

All three orthologues had similar expression patterns in Royal and RE2. This was not unexpected as RE2 is a 5-azaC treated early flowering derivative line of Royal.

Demethylation can result in movement of transposons and thereby change the DNA sequence of a cell. The differences between Royal and RE2 line at the DNA level are unknown, however (Fieldes and Harvey, 2004; House, 2010). *ELF4* orthologue expression was similar between Royal and RE2, which indicates that demethylation or changes to the DNA sequences did not occur at these loci. Thus, changes in *ELF4* orthologue expression was likely not responsible for the early flowering trait observed in RE2 compared to Royal.

The occurrence of peak expression on the 34th day after seeding, in both Royal and RE2, is assumed to be related to apical dominance since apical dominance controls branching in plants (Pan, 2001a). Royal and RE2 have a quite different phenotype compared with CDC Sorrel and CDC Bethune. CDC cultivars are taller, with a greater height to the first branch and appear to be less branched, while Royal and RE2 are much shorter with a lower height to the first branch and appeared to be more branched. *ELF4* expression in the shoot tip of the main stem is thought to inhibit flower formation. As lateral buds would also be developing during this stage of growth, a mechanism to prevent the development of flowers in the side branches would also be required. Thus it was reasonable to assume that the significant increase of *ELF4* during 30th to 34th day after seeding was to inhibit flower development instead of lateral bud formation in both Royal and RE2.

Even though the expression level of *ELF4* orthologues were at a low level the ELF4 orthologue expression patterns were similar between CDC Sorrel and CDC Bethune, as well as between Royal and RE2. This was as expected due to the high similarity among the three orthologues. However, the expression patterns in Royal and RE2 are different

from those in the CDC cultivars. This might be caused by the difference in their genetic background.

4.6 Future Work

In the future, this study can be improved by the following six approaches. Firstly, in the tissue collection process, apical meristems should be isolated precisely from other tissues. As a reference gene, *GAPDH* is stably expressed in both apical and medium stem tissues (Huis et al., 2010). Thus the amount of *GAPDH* mRNA present would be variable, especially compared to *ELF4,* due to the presence of different amounts of non-meristematic tissues in the collected tissues. Secondly, tissue collection and RNA extraction should be conducted in the dark cycle, since *ELF4* expression is the highest during the night hours. Thirdly, the interval between RNA extractions could be shortened to two days, thereby increasing the accuracy of expression pattern examination. Fourthly, RNA extraction could be started earlier, such as three days after emergence. For early-flowering lines such as RE2, RNA extraction started 14 days after seeding and could have resulted in missing gene expression information during its earlier stages of development. Fifthly, staggered planting can be applied to align flowering times in the chamber, which will also increase the accuracy of the experiment. Sixthly, as *ELF4* is co-repressing transcriptional regulation of ARABIDOPSIS PSEUDO-RESPONSE REGULATOR 9 (APRR9) together with *EARLY FLOWERING 3* (*ELF3)* and *LUX,* it would be more comprehensive to include *ELF3* and *LUX* in the expression study. Finally, more cultivars/lines should be involved so that we can further understand the mechanism of *ELF4* gene expression in flax flowering time analyses more comprehensively.

CHAPTER 5

5. Flowering time in 5-azacytidine mutant and hybrid populations of oilseed flax cultivars

5.1 Abstract

The short growing season of the northern Prairies is a major limitation to growing oilseed flax in Saskatchewan. Reducing the length of the life cycle of flax is a major strategy to expand the acreage where flax can be grown successfully in the Canadian prairies. 5-azaC is a potent growth inhibitor, which induces early flowering and dwarfism in oilseed flax. In this study, the effects of 5-azaC were examined in two populations of a widely grown flax cultivar CDC Sorrel. The hybrid population was derived from crosses between CDC Sorrel and three 5-azaC induced early flowering M_9 genotypes (RE1, RE2 and RE3), and the mutant population was derived by treating germinating CDC Sorrel seeds with 5-azaC. Field tests were conducted for two growing seasons at the Kernen Crop Research Farm (KCRF), University of Saskatchewan. Evaluation of phenotypes showed early flowering segregants in the hybrid populations. However, no significant difference was observed in the flowering time of CDC Sorrel and 5-azaC treated CDC Sorrel M_2 and M_3 populations.

5.2 Introduction

Historically, crop yields in the Canadian Prairies have been improved by adapting the lifecycle length with balanced growth and yield (Bueckert and Clarke, 2013). Generally crop plants adapted to the Northern Prairies are early flowering with a shorter vegetative phase but a longer reproductive phase (flowering to seed maturation). This is because early-flowering/maturing crops can avoid being injured by abiotic stresses such as seasonal heat stress and first fall frosts. It was found that time to flowering and seed yield

is negatively related in accessions from Canadian flax core collection (Zhang et al., 2014). Early flowering was related to higher seed yield in the core collection. Moreover, Growing Degree Days Maturity (GDDM) is highly but negatively related to canopy absorption and grain yield in the Canadian flax core collection (Zhang et al., 2014). However, in this germplasm collection a subset of the flax world collection representing the geographic and phenotypic diversity of flax, many accessions were not adapted to the climatic conditions of the Canadian Prairies.

Treating germinating flax seeds with 5-azaC affected plant height, branching, tillering and/or flowering age (Fieldes, 1994; Fieldes and Amyot, 1999). The research carried out by Dr. Fieldes (Wilfred Laurier University, Waterloo, Canada) showed that DNA hypomethylation could be observed five to nine generations beyond the treatment generation (Fieldes and Harvey, 2004). The early-flowering genotypes of flax used in this study were derived from plants treated once as germinating seedlings with 5-azaC in 1990 (Fieldes, 1994). It was found that some early flowering genotypes induced by 5-azaC flowered 7-13 days earlier than the untreated controls, which was due to an accelerated vegetative stage (Fieldes and Harvey, 2004). Flowering age was more variable among the first progeny generation of the treated plants (Fieldes, 1994). However, the stability of the early-flowering trait under field conditions was unknown since this study was conducted under a greenhouse environment.

CDC Sorrel is a large brown seeded, late maturing cultivar with superior oil quality and grain yield equal to CDC Bethune, which is the yield standard and another popularly grown cultivar in Saskatchewan (Rowland et al., 2002; Saskatchewan Ministry of

Agriculture, 2014). In this study, crosses between CDC Sorrel and Royal mutant genotypes were conducted:

a) To introgress the early flowering trait from the RE genotypes by crossing with CDC Sorrel a popularly grown cultivar in Saskatchewan and identify early flowering phenotypes in the F_2 and F_3 of the above crosses under field tests at 53^{o}N in Saskatchewan;
b) To generate early flowering variants of CDC Sorrel using 5-azaC treatment of germinating seeds and assess days to flowering, days to maturity and height of the M_2 to M_3 conducted at 53^{o}N field tests in Saskatchewan
c) To test the stability of the early flowering trait in three 5-azaC derived genotypes RE1, RE2 and RE3 conducted at 53° N field tests in Saskatchewan.

5.3 Materials and methods

5.3.1 Hybrid population

5.3.1.1 Progeny-row populations

The original germplasm used in this study included Royal genotypes (Royal, RE1, RE2, RE3) and CDC Sorrel. RE1, RE2 and RE3 used in this study were derived from plants treated once as germinating seedlings with 5-azaC in 1990 (Fieldes, 1994). The Royal genotypes were kindly provided by Dr. Mary Anne Fieldes (Wilfrid Laurier University, Waterloo, Canada).

Reciprocal crosses were conducted between Royal genotypes (Royal, RE1, RE2, RE3) and CDC Sorrel. CDC Sorrel was used as a parent in crosses with the Royal genotypes for its large seed size, which is often related to higher yield (Soto-Cerda et al., 2014). However, it is a late maturing cultivar. The F_1 generation was grown in a growth chamber

at National Research Council of Canada, Saskatoon and F_2 seed harvested from individual F_1 plants.

F_2 progeny of two F_1 plants from each of the four different reciprocal crosses were increased and grown in rows at the Kernen Crop Research Farm (KCRF) in Saskatoon, Saskatchewan, Canada (Lat 52°09'N). Due to limited seed availability, F_2 rows were seeded on June 2nd 2012 in a type 2 Modified Augmented Design (MAD) design.

A type 2 MAD design is commonly used in the beginning stage of a breeding program when breeders usually do not have an adequate amount of seed for replicated field tests (Lin and Poushinsky, 1985). Considering seed availability, there was no replication of the test rows, while there were a number of replications of the control plots and control subplots. Control plots were used to estimate additive soil variation, and control subplots were used to estimate non-additive soil variation (You et al., 2013). All test genotypes, control plots and control subplots were seeded as single row plots.

In a type 2 MAD design control plots are used to assess if the heterogeneity in soil occurs along rows and/or columns and also to determine the whole plot error. Subplot controls determine the row × column interaction and the subplot error, which indicates the combined effect of a row and a column. Significant row and column effects could mean there is gradual change in soil structure, it is considered as additive effect. Significant row × column interaction would infer that more isolated changes are occurring in the soil, which is not consistent throughout the field.

In this study, all of the parental genotypes including CDC Sorrel and early flowering Royal genotypes (CDC Sorrel, RE1, RE2 and RE3) were seeded in both control plots and control subplots. Royal was seeded as a test entry in F_2's field test. Each row was 3.66 m

long, seeded with 2.5 g of seeds (350-400 seeds), with 0.19 m spacing between each row. Sixteen test genotypes were randomly distributed in this population (see an illustration of field layout of F_2 field test in appendix II.). In total there were 48 plots for the F_2 population including 12 F_2 rows and 32 check rows. Each row was scored for 5% flowering (DTF5%; from sowing to 5% of the plants in a row flowering), maturity date (DTM; from sowing to 80% capsule maturity, i.e. brown bolls of the plot), and height (measurement of the uppermost plant part to ground, at capsule maturity) based on the average performance of the plants in a row. Within each row, selection strategy for early flowering was tagging 50 earliest-flowering individual plants from each row with the date of flowering.

However, a number of selected F_2 generation plants had insufficient seed. Only 229 out of 720 selected F_2 plants were seeded on May 31st as F_3 progeny rows in a type 2 MAD design at the KCRF in 2013. Each row was derived from an individual F_2 plant and was seeded in a 1.22 m single-row plot with 0.19 m spacing between each row. CDC Sorrel, Royal, RE1, RE2 and RE3 were seeded as control plots and control subplots. 229 test genotypes were randomly distributed in this population (see an illustration of field layout of F_3 field test in appendix III). Each row was scored for days to 5% flowering (DTF5%), days to maturity (DTM) and height.

5.3.1.2 Bulk hybrid population

A bulk population of F_3 was also seeded in 2013. Each plot was seeded with bulked seeds from one entire row of 2012 field test. This population was in a lattice design with three replications for each test genotype. In each plot there were six rows with 0.19 m spacing between each row. Sown length was 2.6 m for each row, which was trimmed to

1.83 m after emergence. Thus the width of one plot was 1.37 m including inter-plot spacing and total area of each plot including inter-plot spacing was 2.51 m^2. Each plot was scored for DTF5%, DTM and height.

5.3.2 Mutant population

5.3.2.1 Mutant progeny-row population

One hundred and fifty CDC Sorrel seeds (M_0) were soaked in 30 ml aqueous solutions of 1.5 mM 5-azaC for 14 hours. 1.5 mM was the highest concentration in Dr. Fieldes' protocol (Fieldes, 1994). The treated CDC Sorrel seeds were sown in soil mixture (Sunshine Mix #3 soil) into 4 L size pots and a growth chamber at NRC-Saskatoon. Chamber settings were the same as those applied to F_1 population.

After 5-azaC treatment, some seed washed away during rinsing which caused seed loss before seeding. In addition, a number of M_1 seeds did not germinate. Thus only 98 mutant M_1 plants were derived from the treatment due to seed loss and non-germination. M_1 seeds were grown in a growth chamber at NRC-Saskatoon. M_2 seeds from these 98 treated genotypes were seeded on June 2nd, 2012 at KCRF in a type 2 MAD. Each genotype was seeded in a 1.83 m single-row plot with 0.19 m spacing between each row. CDC Bethune, CDC Sorrel, and Royal genotypes were seeded as the control plots and control subplots. Ninety-eight test genotypes were randomly distributed in this population (see an illustration of field layout of M_2 field test in appendix IV). Including all control plots and control subplots, there were 117 rows in the M_2 field test in total. Each row was scored for DTF5%, DTM and height. One hundred earliest flowering individual plants from the treated CDC Sorrel rows (test genotypes) were selected and harvested separately.

Due to the fact that a few selected plants lost their tags and a few of them did not have enough seeds to be seeded as a row in the M_3 population, 78 out of 100 selected individual plants from M_2 population were seeded as an M_3 population on May 31st, 2013 in a type 2 MAD design. Each row was 1.22m long with 0.19m spacing within each row. In total there were 125 rows in M_3 field test including CDC Sorrel, CDC Bethune, Royal, RE1, RE2 and RE3 as control plots and control subplots. Seventy-eight test genotypes were randomly distributed in this population (see an illustration the field layout of M_3 field test in Appendix V). Each row was scored for DTF5%, DTM and height.

5.3.2.2 Bulk mutant population of M_3

A bulk population of M_3 was seeded on May 31st, 2013. Each plot was seeded with bulked seeds from the entire progeny row of M_2 population. This population was planted in a lattice design with three replications for each test genotype. In each plot there were six rows with 0.19 m spacing between each row. Sown length was 2.6 m for each row, which was trimmed to 1.83 m after emergence. Thus the width of one plot was 1.37 m including inter-plot spacing and total area of each plot including inter-plot spacing was 2.51 m^2. Each plot was scored for DTF5%, DTM and height.

5.3.3 Data analysis

ANOVA analyses were conducted for traits (DTF5%, DTM, and Height) in F_2 and F_3 populations, respectively. Appendix IX and X showed that the M_2 and M_3 population field tests soil heterogeneity existed in both control plots and control subplots. Thus, adjustments were made to account for the effect of soil heterogeneity accordingly.

In type 2 MAD design analysis, there are two common methods used to adjust observations: Method 1(M1) and Method 3 (M3). M1 is based on row and column effects

of plot controls and M3 is based on the regression of test plots on the plot control (Lin and Poushinsky, 1983; Lin and Poushinsky, 1985; Lin and Voldeng, 1989). Recently, You et al. proposed another M1+M3 method to adjust observations that combined M1 and M3 (You et al., 2013). A pipeline for type 2 MAD design analysis (which can be obtained from *http://probes.pw.usda.gov/bioinformatics_tools/MADPipeline/index.html*) was introduced to determine which method (M1, M3 or M1+M3) was appropriate to estimate soil variation across the field (You et al., 2013). In this pipeline, Relative efficiency value (RE) of adjusted versus unadjusted observations was evaluated. The RE was a ratio of unadjusted values to that of the adjusted values of pooled variance within both control plots and control subplots (You et al., 2013). In step 1 (data conversion), raw data (had to have 12 columns with a header line) was converted to the format that can be recognized in step 2. In step 2 PROC SQL and PROC GLM were used for ANOVA of control plots and control subplots in. There were three processes in step 3. Firstly, results of ANOVA from step two were summarized. Secondly, necessary statistics were calculated to adjust the raw data of test genotypes and checks. Thirdly, RE values of different adjustment were estimated for selection of the most appropriate adjustment method(You et al., 2013). Step 1 and 3 were conducted in Perl 5 (Perl.org) and step 2 was conducted in SAS 9.0 (SAS Institute Inc., Cary, NC, USA.).

Pearson Correlation tests of major traits (DTF5%, DTM, Height, TSW) were conducted for the F_3 and M_3 bulked populations in SAS 9.0 (SAS Institute Inc., Cary, NC, USA.).

5.4 Results

5.4.1 F_2 population

Data from 2012 and 2013 field tests were adjusted based on the RE value of each trait in each year (See Appendix VIII). DTF 5% was adjusted with Method 3 since there was significant Row × Column effects which indicated that the heterogeneity exited in soil was inconsistent. After the adjustment, variations were observed in all four traits (DTF5%, DTM, Height and TSW) in F_2 population (see Figure 5.1). Figure 5.1 shows the four traits (DTF5%, DTM, height and TSW) examined in the F_2 generation. Variation in all four traits (DTF5%, DTM, height and TSW) was observed under field conditions. Most F_2 progeny rows presented intermediate phenotypes compared with parental genotypes CDC Sorrel, Royal, RE1, RE2 and RE3. For example, DTF 5% (Figure 5.1.a), parental genotype CDC Sorrel flowered 57.0 days after seeding. Royal, RE1, RE2 and RE3 flowered 53.0, 47.9, 46.2 and 48.2 days respectively after seeding, while their progeny genotypes flowered 53.8, 51.3, 51.3 and 50.8 days after seeding. Although still segregating, the progenies from the CDC Sorrel/Royal-1 crosses showed a transgressive (earlier flowering/maturing) phenotype in the field test in 2012.

DTM (Figure 5.1.b) of F_2 population was adjusted with Method 3 since there was significant Row × Column effects. F_2 progenies also exhibited variations. Parental genotypes CDC Sorrel, Royal, RE1, RE2 and RE3 matured 104.8, 106.0, 100.8, 92.1, 94.9 days after seeding respectively, while progeny genotypes matured 91.2-112.5 days after seeding.

Parental genotypes CDC Sorrel, Royal, RE1, RE2 and RE3 were 68.0, 47.5, 49.8, 40.4 and 46.8 cm in height respectively (Figure 5.1.c). Among the progeny genotypes, the

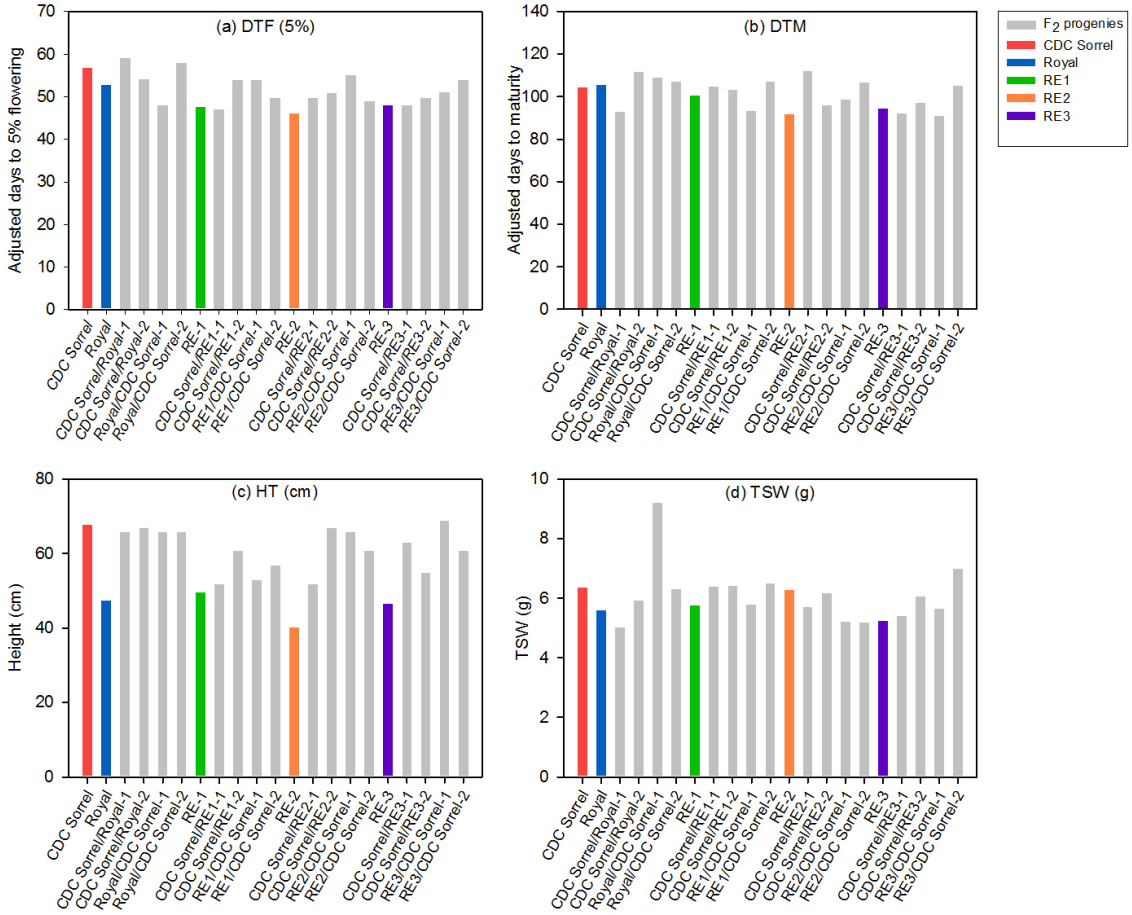


Figure 5.1 Agronomic traits of F_2 single-row progeny population.
Colored bars indicate parental genotypes CDC Sorrel, Royal, RE1, RE2 and RE3. Grey bars indicate progeny genotypes. As was suggested by relative efficiency (RE) values (Appendix VIII), both Days to 5% flowering and Days to maturity were adjusted by M3. However, it is unnecessary to adjust Height and TSW.

shortest progenies were RE1/CDC Sorrel-1 and RE2/CDC Sorrel-1, both were only 52 cm, while the tallest progeny CDC Sorrel/RE3-1 was 69.0 cm, even taller than its parent CDC Sorrel.

For TSW (Figure 5.1.d), parental genotypes CDC Sorrel, Royal, RE1, RE2 and RE3 were 6.38 g, 5.62g, 5.78g, 6.31g and 5.26g, respectively. Progeny rows range from 5.04 g to 9.24 g. The heaviest test genotype was Royal/CDC Sorrel-1. Plant stand of this population was compared but no significant difference was detected. Thus it is reasonable to assume that low plant number was not the cause on an increase in seed weight in the genotype with the largest seed weight.

In the F_2 population, the performance of progeny lines from reciprocal crosses was similar for all four traits examined.

5.4.2 F_3 single-plant derived population

In the F_3 single-plant derived progeny population, DTF 5% was adjusted with Method 3 since there was significant Row × Column effects which indicated that there was heterogeneity exhibited in soil conditions across the field test. After the adjustment, variation existed for DTF 5% (Figure 5.2). Among all parental genotypes, RE2 was the earliest flowering genotypes. The parental genotypes CDC Sorrel, Royal, RE1, RE2 and RE3 flowered 51.5, 47.4, 43.0, 39.5, 45.2 days after seeding, while the progeny F_3 progenies flowered 34.6~53.6 days after seeding. Due to the selection for early flowering in F_2 generation, all segregants flowered earlier than the late-flowering parent CDC Sorrel except for two rows in CDC Sorrel/RE1 (Figure 5.2.b), many exhibited a transgressive phenotype: flowering earlier than the earlier-flowering parents (Royal, RE1, RE2 and RE3).

For DTM (Figure 5.3), the parental genotypes CDC Sorrel, Royal, RE1, RE2 and RE3 matured 100.0, 94.0, 101.0, 94.0 and 93.0 days after seeding, respectively, while F_3 progenies matured from 88.3 to 101.3 days after seeding. Many segregants matured earlier than both their parents.

For height (Figure 5.4), the parental genotypes CDC Sorrel, Royal, RE1, RE2 and RE3 were 71.9, 60.1, 57.4, 48.6 and 53.8 cm respectively, while height of F_3 progenies ranged from 45-81 cm. The shortest genotype was 2012SorrelxEFf2's104-5, derived from cross between RE1 and CDC Sorrel, and the tallest genotype was 2012SorrelxEFf2's112-16, derived from cross between Royal and CDC Sorrel.

For TSW (Figure 5.5), the parental genotypes CDC Sorrel, Royal, RE1, RE2 and RE3 were 6.52 g, 6.16 g, 6.75 g, 6.39 g and 5.75 g, respectively, while F_3 progenies ranged from 5.23 g-8.11 g. Among the F_3 progeny genotypes, 2012 SorrelxEFf2's104-25 has the largest seed size, which was derived from cross between RE1 and CDC Sorrel.

Pearson Correlation tests suggested that (Table 5.1), all measured traits (DTF5%, DTM, TSW and HT) were significantly correlated with each other except for Height vs TSW, DTF5% and TSW were negatively but significantly correlated with each other, indicating that early flowering was associated with a larger seed size and a smaller plant height in these populations.

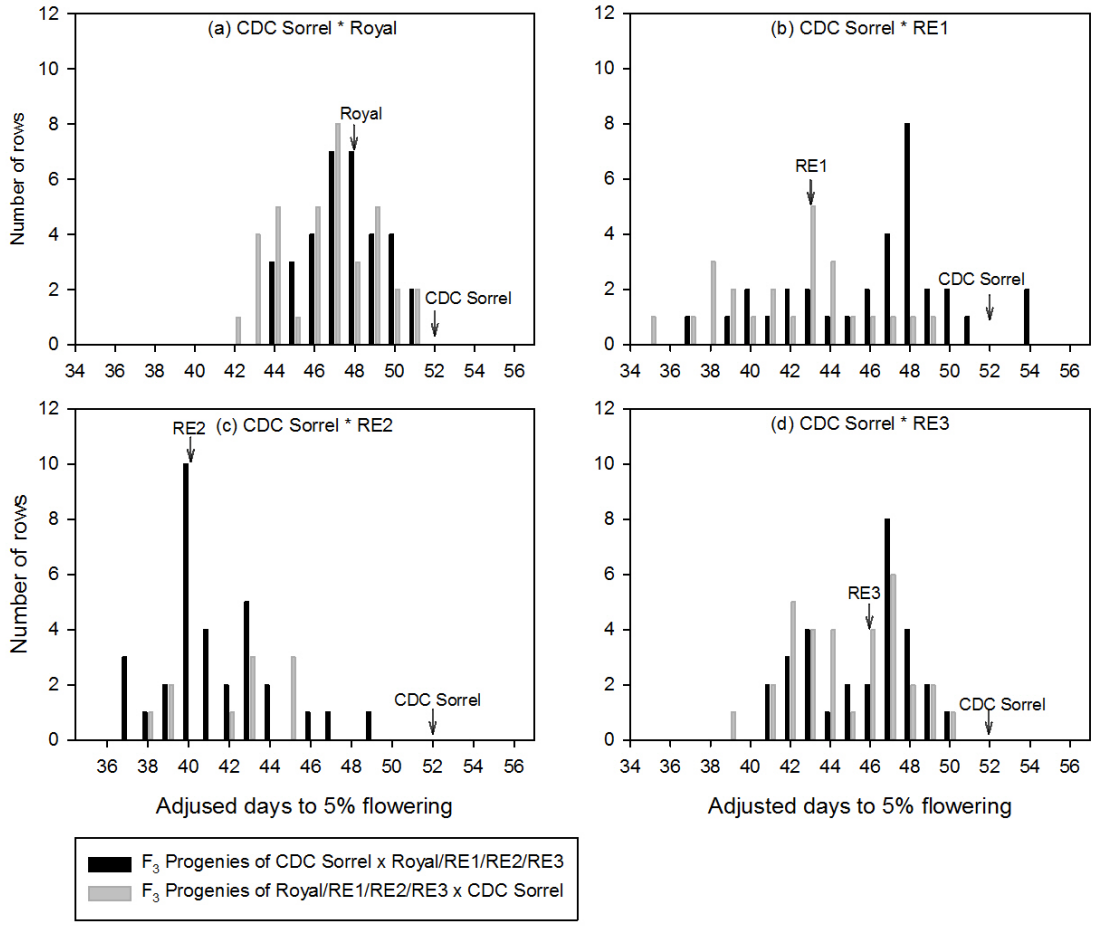


Figure 5.2 DTF5% of selected F_3 single-plant derived progeny populations.
Each of the four graphs refers to progeny genotypes derived from different crosses: (a) CDC Sorrel x Royal and Royal x CDC Sorrel, (b) CDC Sorrel x RE1 and RE1 x CDC Sorrel, (c) CDC Sorrel x RE2 and RE2 x CDC Sorrel, and (d) CDC Sorrel x RE3 and RE3 x CDC Sorrel. Compared with intermediate phenotypes, more transgressive phenotypes were observed in this F_3 population than the F_2 population. Even though the progeny genotypes were from different crosses, a number of progeny genotypes flowered earlier than both their parental genotypes.

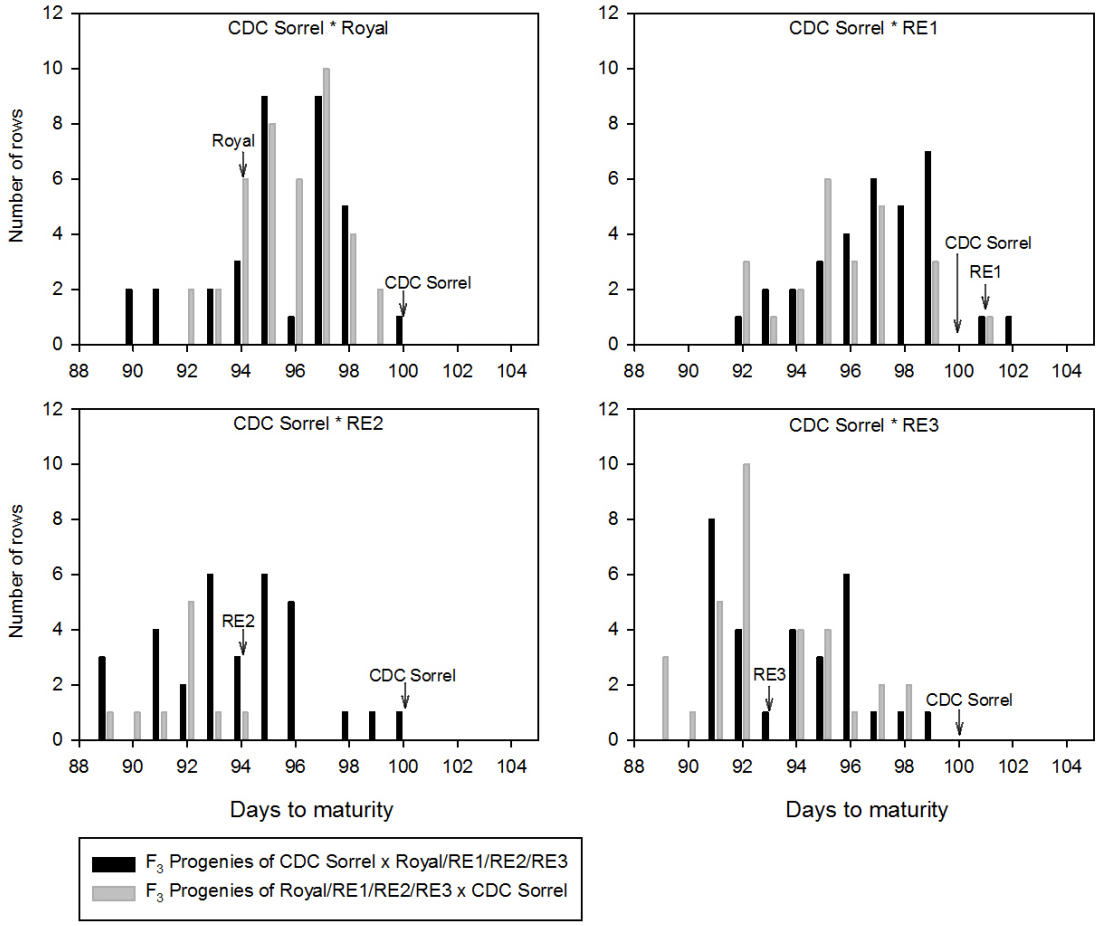


Figure 5.3 DTM of selected F_3 single-plant derived progeny populations.
Each of the four graphs refers to progeny genotypes derived from different crosses: (a) CDC Sorrel x Royal and Royal x CDC Sorrel, (b) CDC Sorrel x RE1 and RE1 x CDC Sorrel, (c) CDC Sorrel x RE2 and RE2 x CDC Sorrel, and (d) CDC Sorrel x RE3 and RE3 x CDC Sorrel. Similar with DTF, when compared with intermediate phenotypes, more transgressive phenotypes were observed in this F_3 population than the F_2 population. Even though the progeny genotypes were from different crosses, a number of progeny genotypes matured earlier than both their parental genotypes.

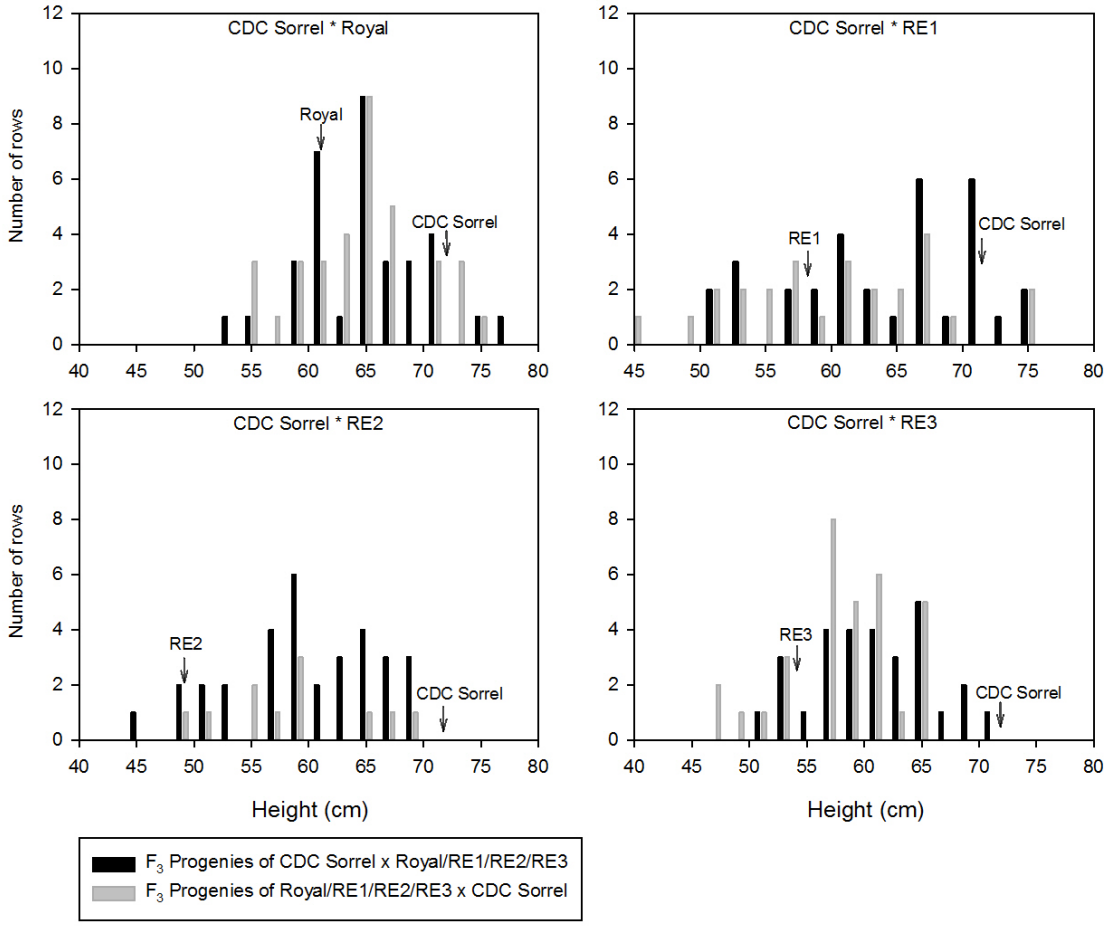


Figure 5.4 Height of selected F_3 single-plant derived progeny population.
Each of the four graphs refers to progeny genotypes derived from different crosses: (a) CDC Sorrel x Royal and Royal x CDC Sorrel, (b) CDC Sorrel x RE1 and RE1 x CDC Sorrel, (c) CDC Sorrel x RE2 and RE2 x CDC Sorrel, and (d) CDC Sorrel x RE3 and RE3 x CDC Sorrel. Variation in height was observed in progeny genotypes derived from different crosses. Transgressive and intermediate phenotypes (taller or shorter than parental genotypes) were observed.

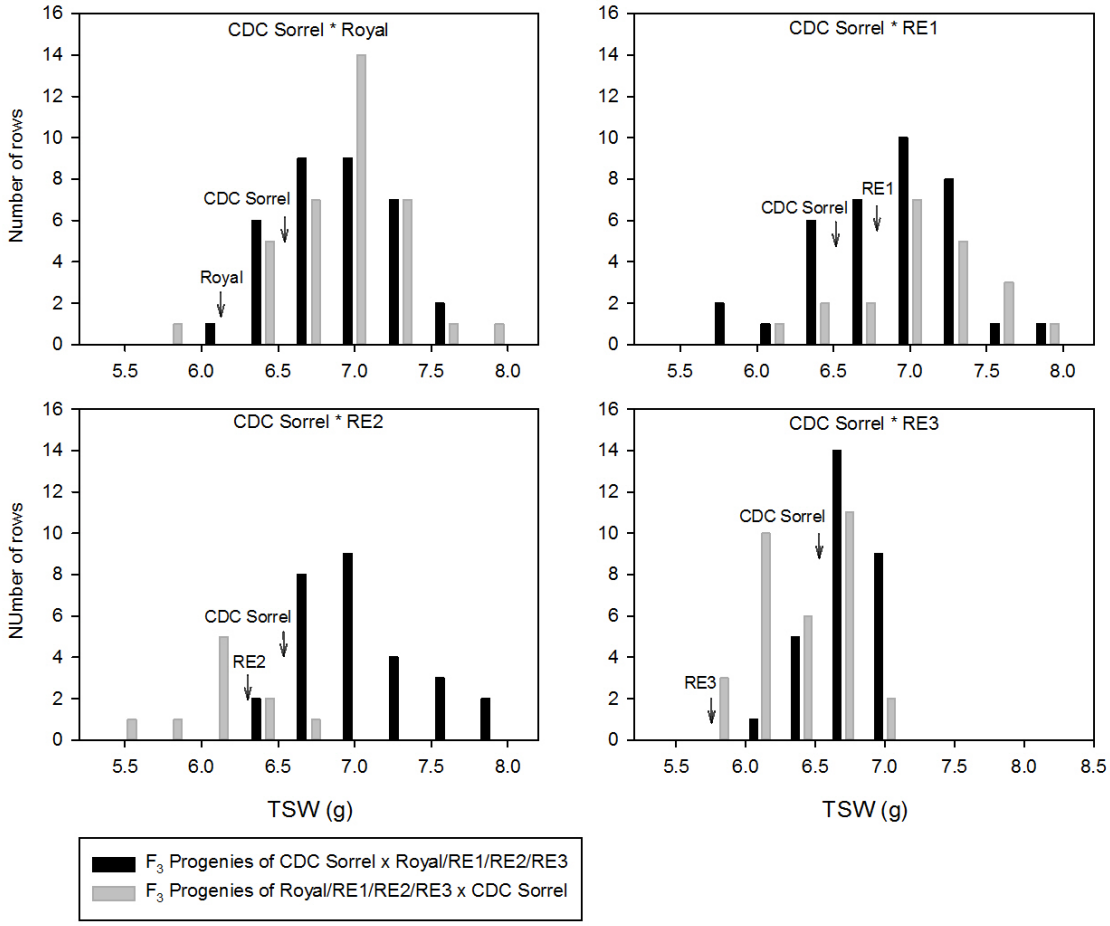


Figure 5.5 TSW of selected F_3 single-plant derived progeny population.
Each of the four graphs refers to progeny genotypes derived from different crosses: (a) CDC Sorrel x Royal and Royal x CDC Sorrel, (b) CDC Sorrel x RE1 and RE1 x CDC Sorrel, (c) CDC Sorrel x RE2 and RE2 x CDC Sorrel, and (d) CDC Sorrel x RE3 and RE3 x CDC Sorrel. Transgressive phenotypes were observed in progeny genotypes derived from different crosses. For progeny genotypes derived from crosses between RE3 and CDC Sorrel, all F_3 test genotypes had larger seed size than its parental genotype RE3. DTF was adjusted by Method 3 since there was Row × Column effects existing.

Table 5.1 Pearson Correlation tests of F_3 rows for DTF5%, DTM, TSW and HT.

	DTF5%	DTM	TSW	HT
DTF5%	1			
DTM	0.45623***	1		
TSW	-0.26089***	0.24512***	1	
HT	0.52141***	0.37925***	-0.03939	1

Significant differences are indicated at three levels: *p<0.05* (*), *p<0.01* (**) and *p<0.001*(***).

5.4.3 Bulk F_3 population

In F_3 bulk population (Figure 5.6.a), parental genotypes CDC Sorrel, Royal, RE1, RE2 and RE3 flowered 51.5, 44.5, 41.0, 44.0, 48.0 days after seeding, respectively, while bulk F_3 plots reached 5% flowering 43.4-48.0 days after seeding. LSD at 0.05 level was 1.79, which indicated that all progeny plots flowered significantly earlier than CDC Sorrel. In addition, the bulk F_3 progenies whose maternal parent was an RE genotype (RE1/RE2/RE3) flowered significantly earlier than bulk F_3 progenies derived from Royal/CDC Sorrel. This indicated that flowering time might be maternally inherited.

Parental genotypes CDC Sorrel, Royal, RE1, RE2 and RE3 matured 100.2, 92.8, 96.3, 92.3 and 90.7 days after seeding, respectively. F_3 progenies reached maturity 91.7-99.7 days after seeding. LSD at 0.05 level was 3.93, which indicated that 11 out of 16 (73.3%) bulk F_3 plots matured significantly earlier than CDC Sorrel.

For height, parental genotypes CDC Sorrel, Royal, RE1, RE2 and RE3 were 68.0, 58.5, 52.3, 54.0 and 47.7 cm respectively, while bulk F_3 plots height ranged from 57.0 to 67.3 cm. LSD at 0.05 level was 6.41, which suggested that 7 out of 16 (43.8%) bulk F_3 plots were significantly shorter than CDC Sorrel.

TSW of parental genotypes CDC Sorrel, Royal, RE1, RE2 and RE3 was 6.62, 6.09, 6.49, 6.18, and 5.57 g, respectively, while bulk F_3 plots ranged from 5.58 to 6.81 g. LSD at 0.05 level was 0.49, which indicated that overall there 6 F_3 progeny plots had a significantly smaller seed size than CDC Sorrel.

Results of Pearson Correlation tests (Table 5.2) indicated that DTF5% was highly and significantly correlated with DTM ($p<0.01$). DTM was significantly correlated with TSW ($p<0.05$). In addition, TSW was significantly correlated with height.

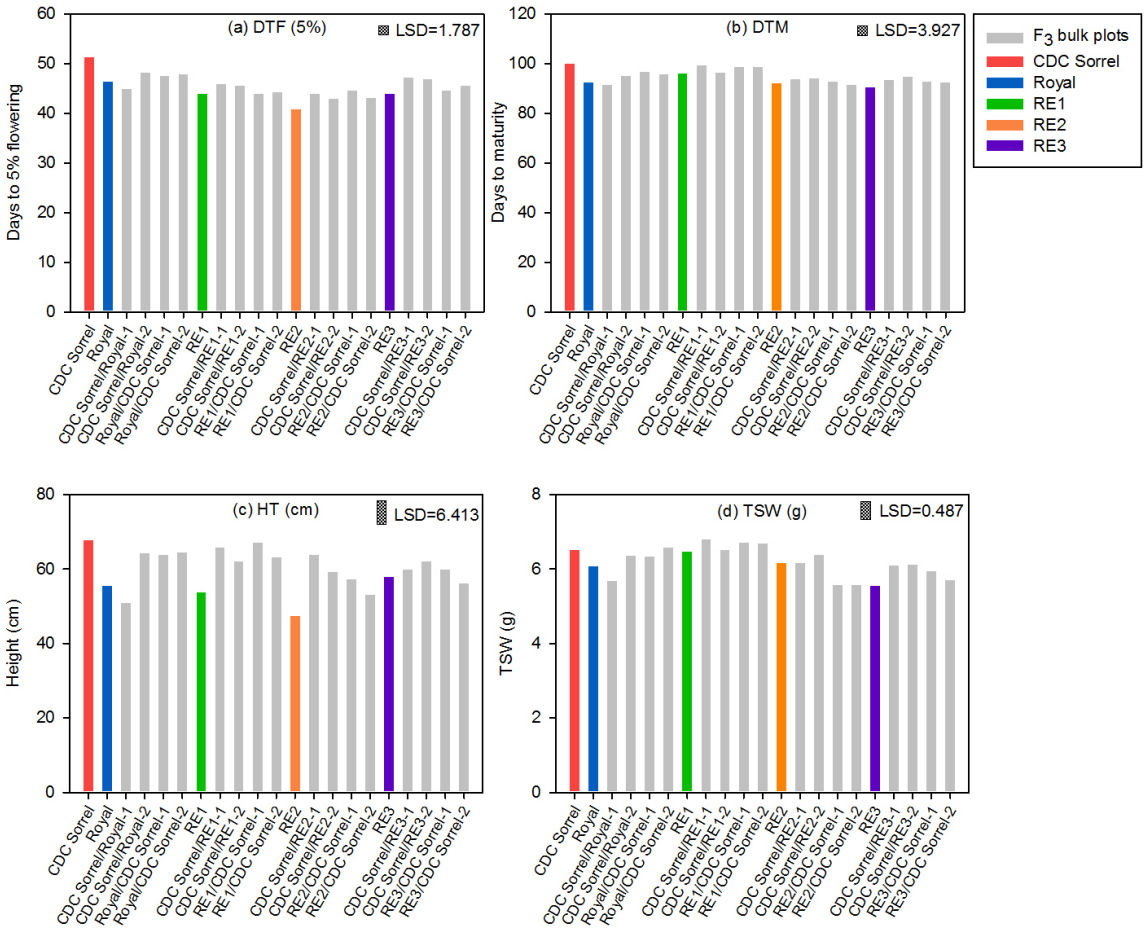


Figure 5.6 Agronomic traits of F_3 bulk population.
Colored bars indicate parental genotypes CDC Sorrel, Royal, RE1, RE2 and RE3. Grey bars indicate bulk F_3 plots. Variation in all four traits was observed in this population. Height of the boxes on the top right corner of each graph stands for the LSD value of each trait on 0.05 level.

Table 5.2 Pearson Correlation tests of bulk F_3 plots for DTF5%, DTM, TSW and HT.

	DTF5%	DTM	TSW	HT
DTF5%	1			
DTM	0.87265***	1		
TSW	0.21992	0.29286*	1	
HT	0.10632	0.18586	0.60339***	1

Significant differences are indicated at three levels: $p<0.05$ (*), $p<0.01$ (**) and $p<0.001$(***).

5.4.4 M_2 population

DTF5%, DTM, height and TSW varied in the M_2 population (Figure 5.7). DTF was adjusted by Method 1+ Method 3 based on the suggestion for best adjustment given by RE values. M_2 genotypes flowered 47.3-62.4 days after seeding, while the original material CDC Sorrel (seeded as control rows) flowered 58.1 days after seeding (Figure 5.7). There were 67 out of 98 M_2 rows (68.4%) flowering earlier than untreated CDC Sorrel. Royal flowered 52.3 days after seeding, while its 5-azaC treated derivatives RE1, RE2 and RE3 flowered 48.6, 48.8 and 51.3 days after seeding in 2012, respectively.

DTM of this population was adjusted by Method2 because of the significant Row × Column effects. M_2 genotypes matured 96.4-110.4 days after seeding, while CDC Sorrel matured 110.1 days after seeding (Figure 5.7.b). There were 42 out of 98 M_2 rows (42.9%) maturing earlier than untreated CDC Sorrel. Royal matured 96.5 days after seeding, while its 5-azaC treatment derivatives RE1, RE2 and RE3 flowered 94.4, 86.9 and 84.8 days after seeding, respectively.

CDC Sorrel treated by 5-azaC ranged from 62 to 83 cm in height, while untreated CDC Sorrel was 73.8 cm (Figure 5.7.c). 60.2% of the M_2 rows were shorter than the original material CDC Sorrel. Royal was 57.8 cm at maturity, while its 5-azaC treatment derivatives were 54.0, 36.0 and 43.0 cm after seeding, respectively.

TSW of M_2 mutant genotypes ranged from 6.32 to 8.12 g, while CDC Sorrel was 6.82 g (Figure 5.7.d). 66.2% of the test genotypes were heavier than untreated CDC Sorrel. Royal had a TSW of 6.13 g, while RE1, RE2 and RE3 was 7.20, 6.20 and 5.96 g, respectively.

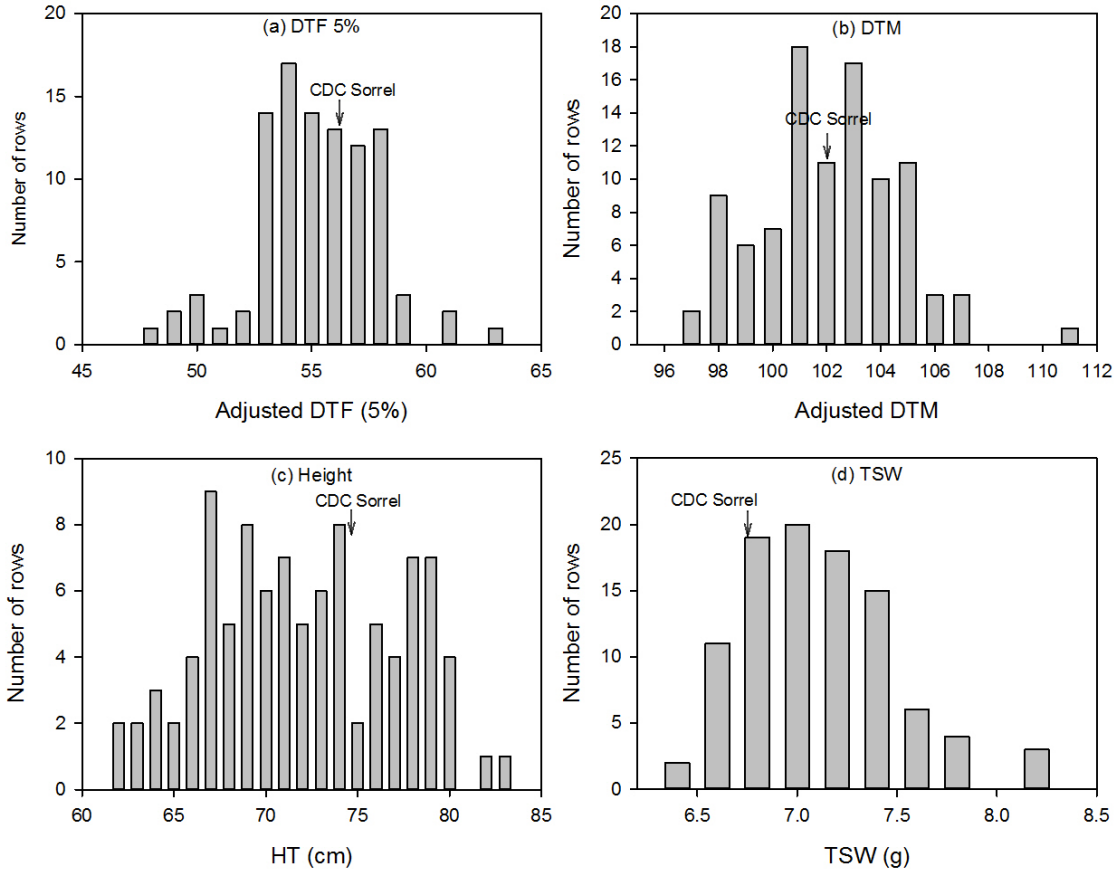


Figure 5.7 Frequency distribution of four major traits (DTF5%, DTM, HT and TSW) (adjusted values for DTF and DTM) in M_2 population.
DTF was adjusted by Method1+ Method3, and DTM was adjusted by Method 3. Variation in all four traits existed in M_2 population. More than half of M_2 test genotypes flowered earlier than their original germplasm CDC Sorrel (a). Earlier maturity, shorter plant height and larger seed size were also observed in this population (b).

5.4.5 M_3 single-plant derived population

M_3 rows in this population flowered 42-54 days after seeding, while CDC Sorrel flowered 50.3 days after seeding. There were 59 out of 78 M_3 rows (75.6%) flowering earlier than its original material CDC Sorrel. Royal flowered 45.6 days after seeding while RE1, RE2 and RE3 flowered 45.3, 41.9 and 43.5 days after seeding, respectively.

For DTM, M_3 rows matured 91-105 days after seeding, while CDC Sorrel matured 98.7 days after seeding. There were 35 out of 78 M_3 rows (44.9%) maturing earlier than CDC Sorrel. Royal matured 92.3 days after seeding, while RE1, RE2 and RE3 matured 100.3, 94.9 and 91.6 days after seeding, respectively.

For height, M_3 rows were ranged from 57 to 81cm, while CDC Sorrel was 74.9cm. There were 41 out of 78 M_3 rows (52.6%) shorter than CDC Sorrel. Royal was 62.4 cm while RE1, RE2 and RE3 were 58.9, 57.1 and 52.9 cm, respectively.

TSW of M_3 mutant genotypes ranged from 6.07 to 7.24 g, while untreated CDC Sorrel was 6.51 g. There were 82.1% of the M_3 test genotypes having larger TSW than the original germplasm CDC Sorrel.

5.4.6 Bulk M_3 population

In bulk M_3 population, CDC Sorrel flowered 50.5 days after seeding, while M_3 bulk plots flowered 50-51.7 days after seeding. LSD at 0.05 level was 1.44, which indicated that there was no significant difference in flowering time (DTF 5%) between untreated CDC Sorrel and 5-azaC treated CDC Sorrel. However, when comparing within the mutant genotypes, five earliest genotypes (12_5azaC_Mutants_217, 12_5azaC_Mutants_131, 12_5azaC_Mutants_138, 12_5azaC_Mutants_167 and 12_5azaC_Mutants_163) flowered significantly earlier than three genotypes which

flowered latest (12_5azaC_Mutants_176, 12_5azaC_Mutants_179 and 12_5azaC_Mutants_135) by 1.7 days.

For days to maturity, CDC Sorrel matured 99.7 days after seeding, while M_3 progenies matured 94.7-100.0 days after seeding. LSD at 0.05 level was 3.9, which indicated that there were bulk plots that matured significantly earlier than CDC Sorrel. There were 6 out of 42 bulk M_3 progeny plots (14.3%) maturing significant earlier than CDC Sorrel.

CDC Sorrel was 66.5cm, while bulk M_3 plots ranged in height from 60.0-71.3 cm. LSD at 0.05 level was 5.2, which indicated that there were progeny plots that were significantly shorter than CDC Sorrel. There were 7.14% of the progeny plots shorter than untreated CDC Sorrel.

TSW of mutant M_3 genotypes ranged from 6.06 to 6.74 g, while untreated CDC Sorrel was 6.25 g. LSD at 0.05 level was 0.39, which indicated that 87.76% of the M_3 genotypes had significantly larger seed size than untreated CDC Sorrel. TSW for Royal was 6.08 g, while that of RE1, RE2 and RE3 was 6.09, 6.12 and 5.54 g, respectively. Based on the LSD value, RE3 had significantly smaller seed size than its original germplasm Royal.

Pearson Correlation tests suggested that (Table 5.3), all measured traits (DTF5%, DTM, TSW and Height) were significantly correlated with each other.

Table 5.3 Pearson Correlation tests in bulk M_3 population for DTF5%, DTM, TSW and HT.

	DTF5%	DTM	TSW	HT
DTF5%	1			
DTM	0.48002***	1		
TSW	0.25386**	0.34496***	1	
HT	0.25993**	0.67093***	0.50256***	1

Significant differences are indicated at three levels: $p<0.05$ (*), $p<0.01$ (**) and $p<0.001$(***).

5.5 Discussion

Compared with parental genotypes in the F_3 single-plant derived progeny population, progeny plots presented intermediate phenotypes, as well as transgressive phenotypes. This indicates that the selection for early flowering conducted at the previous F_2 generation influenced the progeny flowering time in the next generation shifting most to be earlier that the parental genotype CDC Sorrel and many earlier than Royal and its early flowering derivatives. A side effect of treating germinating seeds with 5-azaC is that the height of plants is shortened plants derived from treated seeds were significantly shorter at maturity than untreated ones (Fieldes, 1994). Comparing with the control plants, the M_2 generation of treated Royal (24 h-treatment) was 20.1% shorter on average (Fieldes, 1994). Figure 5.4 shows the comparison of height among all progeny plots. Overall, CDC Sorrel/RE2 is the shortest group. Figure 5.5 shows the comparison of TSW in the F_3 progeny plots. The same with DTF, progenies also present intermediate phenotypes and transgressive phenotypes.

In the bulk F_3 population, all progeny plots were flowering significantly earlier than CDC Sorrel (Figure 5.6.a). CDC Sorrel flowers 51.5 days after seeding, while F_3 test plots flowered between 43.0 to 48.3 days. The earliest flowering genotype in this bulk F_3 population is 12_SorrelxEFf2's_121 (bulked), which was derived from cross between CDC Sorrel and RE2. This indicates that the early flowering trait has been successfully introgressed in to the offspring and is stable in under field conditions. To investigate whether the advance in flowering time was induced by Royal (the genetic background) or the mutation induced in Royal by 5-azaC, progenies derived from different parents (Royal and its early derivative genotype RE2) were compared (Figure 5.6). It was found

that, bulk F_3 progenies of RE2/CDC Sorrel flowered (44.7 and 43.3 days after seeding) significantly earlier than those of Royal/CDC Sorrel (47.7, 48.0 days after seeding) based on an LSD value of 1.787. In addition, all bulk F_3 progeny where maternal parent was an RE genotypes (RE1/2/3) flowered significantly earlier than bulk F_3 progenies of Royal/CDC Sorrel. This indicates that the advancement in flowering time may be in this material may be maternally inherited was also reported by Manggoel and Uguru (2012). For the bulk F_3 progeny where maternal parent was CDC Sorrel, flowering time and maturity time varied. A correlation analysis for the relationship between TSW and plant stand showed no significant correlation, indicating the larger seed size was due to genotype and not reduced competition within the plot.

Meanwhile, in the F_3 bulked population, within the F_3 progeny plots derived from crosses between RE2 and CDC Sorrel there was no significant difference in flowering time, which indicate that the advancement is less variable.

In the mutant population, 68.4% M_2 rows flowered earlier than untreated CDC Sorrel (Figure 5.11), while 42.9% matured earlier than untreated CDC Sorrel. In M_3 single-plant derived population, 75.6% progenies are flowering earlier than CDC Sorrel. Bulk M_3 test was done to determine if 5-azaC treatment caused phenotypic changes which were significantly greater than environmental variation of a field test. In the bulk M_3 population, untreated CDC Sorrel flowered 50.5 days after seeding, while bulk M_3 test plots flowered 50.0 to 51.7 days after seeding. LSD at 0.05 level was 1.439. Thus, the results reveal that flowering time of bulk M_3 plots is not significantly different from untreated CDC Sorrel. Untreated CDC Sorrel matures 99.7 days after seeding, while bulk M_3 test plots matures 94.7 to 100.3 days after seeding. LSD at 0.05 level was 3.921,

which indicates that there were 6 out of 42 M_3 test plots that matured significantly earlier than untreated CDC Sorrel. In addition, significant differences existed in plant height. LSD at 0.05 level was 4.73 for height. Untreated CDC Sorrel is 66. 2cm, while height of M_3 test plots ranges from 59.2 cm to 70.3 cm. Thus among all M_3 test plots, there are three genotypes that were significantly shorter than untreated CDC Sorrel.

The following experiments can be done to determine the reason that flowering time of 5-azaC treated CDC Sorrel was not advanced. First, Royal can be treated with 5-azaC and then directly scored for its flowering time under field conditions. If significantly earlier flowering time can be observed among its mutant genotypes, it is reasonable to conclude that 5-azaC treatment is applicable in inducing early flowering trait, while in this experiment CDC Sorrel is not the ideal material to be treated. Second, if no significant early flowering is observed among its mutant genotypes, prior to field tests, flowering time of CDC Sorrel can be assessed under controlled environment (i.e. growth chamber, greenhouse) for several generations (variants inbred) before and after 5-azaC treatment. In this study 5-zazcytidine treatment concentration was 1.5mM, which was the highest concentration used in Fieldes' study that causes the most significant phenotypic changes among all concentrations (0, 0.5 mM, 1.0mM, and 1.5 mM) (Fieldes, 1994).

CHAPTER 6

6. General discussion, conclusions and future research

6.1 General discussion

Canada is leading flax production worldwide. However, flax acreage in Canada is still limited due to the fact that flax is not well adapted to the short growing season in the northern Prairies. Advancing flowering and maturity time to shorten the life cycle of flax plants prevents the flax crop from suffering frost damage. Thus, breeding new flax cultivars that are adapted to the climate in the northern prairies is one solution to this problem.

This project focused on understanding flax flowering time, which is necessary to breed northern-adapted flax cultivars. Three studies were conducted to achieve this goal: 1) photoperiod sensitivity of Canadian flax cultivars and 5-azaC treated early flowering derivatives; 2) *ELF4* gene expression pattern in flax; and 3) flowering time in 5-azaC mutants of oilseed flax cultivars.

Photoperiod sensitivity of flax was not well understood prior to this thesis study. A weekly reciprocal transfer experiment between a LD chamber and a SD chamber was designed to estimate the response to photoperiod changes and the length of each phase in different flax cultivars and genotypes. This experiment showed that the vegetative stage of a flax plant can be divided into three phases: a basic vegetative phase, a photoperiod sensitive phase and a post photoperiod-sensitive phase. Results showed that all cultivars and genotypes had different flowering times and they were all sensitive to photoperiod changes at different levels. Moreover, the timing of the photoperiod sensitive phase was closely related to flowering time in flax. Specifically, three mutant genotypes RE1, RE2 and RE3 flowered earlier than CDC Sorrel, CDC Bethune, Flanders, Prairie Thunder and

Royal, while RE2 was the least photoperiod sensitive genotype. RE2 exhibited significantly stable early flowering in both the controlled environments and field tests (see Chapter 5).

In the second experiment, the expression pattern of a flowering-related gene *ELF4* was examined. In Arabidopsis, the expression of *ELF4* inhibits the onset of the reproductive stage and, as a result, delays flowering time. To investigate whether its expression was reduced in the early flowering genotypes of oilseed flax, in this study, sampling was conducted every four days starting from 10 days after emergence until flowering began. RT-qPCR was conducted using c-DNA as the template. The expression pattern of *ELF4* in Royal and RE2 was highly similar with each other, and RE2 was determined to be a hypomethylated mutant genotype of Royal (by Dr. M. Fieldes). Based on these observations, it is reasonable to assume that hypomethylation did not occur at the *ELF4* locus.

In the field study two populations were generated. The first population was derived from treating germinating seeds of CDC Sorrel with 5-azaC, and the second population was derived from crosses between CDC Sorrel and Royal, CDC Sorrel and RE1, CDC Sorrel and RE2, as well as CDC Sorrel and RE3. In the M_3 population, no significant difference in flowering time was found. However, in the bulk M_3 population, it was found that 14.3% of the progeny plots matured earlier than untreated CDC Sorrel. In the hybrid population, all F_3 progeny genotypes exhibited significantly earlier flowering time than CDC Sorrel under field conditions, which indicated that the earlier flowering time trait had been successfully introgressed into CDC Sorrel background. In addition, all bulk F_3 progeny where maternal parent was an RE genotypes (RE1/2/3) flowered significantly

earlier than bulk F_3 progenies of Royal/CDC Sorrel. This indicates that the advancement in flowering time may be maternally inherited a result supported by previous studies in cowpea that demonstrated that offspring plants derived from crosses whose maternal parent was a short day (SD) accession would flower only under conditions where a short critical day length was reached (Manggoel and Uguru, 2012). Similar results were also found for offspring derived from crosses whose maternal parent was a day neutral (DN) accession flowering time of the F_1 progeny was within the DN parental flowering time (Manggoel and Uguru, 2012). For the bulk F_3 progeny where maternal parent was CDC Sorrel, flowering time and maturity time varied. For example, the flowering time of F_3 progeny plots of CDC Sorrel/Royal-1 (45.0 days after seeding) and CDC Sorrel/Royal-2 (48.3 days after seeding) was significantly different. In Addition, CDC Sorrel/RE2 flowered significantly earlier (44.0 and 43.0 days after seeding) than all other progeny plots.

The early flowering derivatives of Royal (RE genotypes) were induced and have always been grown under controlled environment (greenhouse), it remained unknown if the early flowering characteristic would be stable under field conditions. Thus, a major finding of the field test was that the early-flowering trait in the RE genotypes was stable under 53° N field tests in Saskatchewan.

All three studies in this thesis provide knowledge for scientists and plant breeders to further understand flowering time in flax, which can be applied to the development of northern-adapted flax cultivars for the Canadian Prairies.

6.2 Novel findings

- All selected flax cultivars and genotypes are photoperiod sensitive, and their response to day length change varied.
- Nodes on the main stem and height to first branch can be used as indicators to predict flowering time in flax.
- Expression patterns of ELF4 orthologues are different in CDC cultivars and Royal/RE2.
- The early-flowering trait from the RE genotypes was successfully introgressed into CDC Sorrel background.
- The early-flowering trait in RE1, RE2 and RE3 was expressed under 53° N field test conditions in Saskatchewan, and these genotypes were significantly earlier the their progenitor Royal. In addition the flowering time of RE1, RE2 and RE3 were stable across the field tests conducted at 53 degrees north latitude in the Canadian Prairies.

6.3 Future research

In the future, this project can be extended to three different aspects. Firstly, identifying the difference in both methylation patterns and DNA sequence changes related to the early flowering trait in the RE genotypes. Secondly, comparing the methylation and expression patterns of genes that control flowering time in these genotypes. Thirdly, advancing the selected early-flowering F_3 lines towards registering an early-flowering and maturing cultivar that is adapted to the climate of the Canadian Prairies.

References

Adams, S. R., S. Pearson and P. Hadley, 1998: An appraisal of the use of reciprocal transfer experiments: assessing the stages of photoperiod sensitivity in chrysanthemum cv. Snowdon (Chrysanthemum morifolium Ramat.). J Exp Bot **49**, 1405-1411.

Akimoto, K., H. Katakami, H. J. Kim, E. Ogawa, C. M. Sano, Y. Wada and H. Sano, 2007: Epigenetic inheritance in rice plants. Ann Bot-London **100**, 205-217.

Alagarswamy, G., D. M. Reddy and G. Swaminathan, 1998: Durations of the photoperiod-sensitive and -insensitive phases of time to panicle initiation in sorghum. Field Crop Res **55**, 1-10.

Altschul, S. F., W. Gish, W. Miller, E. W. Myers and D. J. Lipman, 1990: Basic Local Alignment Search Tool. J Mol Biol **215**, 403-410.

Amado, L., R. Abranches, N. Neves and W. Viegas, 1997: Development-dependent inheritance of 5-azacytidine-induced epimutations in triticale: analysis of rDNA expression patterns. Chromosome Res **5**, 445-450.

Anbessa, Y., T. Warkentin, R. Bueckert and A. Vandenberg, 2007: Short internode, double podding and early flowering effects on maturity and other agronomic characters in chickpea. Field Crop Res **102**, 43-50.

Anonymous, 2002: Growing Flax: Production, Management and Diagnostic Guide. Flax Council of Canada; Saskatchewan Flax Development Commission, Winnipeg; Saskatoon.

Ausín, I., C. Alonso-Blanco and J. M. Martínez-Zapater, 2005: Environmental regulation of flowering. Int J Dev Biol **49**, 689-705.

Bernier, G. and C. Périlleux, 2005: A physiological overview of the genetics of flowering time control. Plant Biotechnol J **3**, 3-16.

Berti, M., S. Fischer, R. Wilckens, F. Hevia and B. Johnson, 2010: Adaptation and genotype x environment interaction of flaxseed (Linum usitatissimum L.) genotypes in South Central Chile. Chilean Journal of Agricultural Research **70**, 345-356.

Bird, A., 2002: DNA methylation patterns and epigenetic memory. Gene Dev **16**, 6-21.

Booker, H., 2014: Canadian Food Inspection Agency - FP2385. **2014**.

Brown, P. T. H., 1989: DNA Methylation in Plants and Its Role in Tissue-Culture. Genome **31**, 717-729.

Bueckert, R. A. and J. M. Clarke, 2013: Review: Annual crop adaptation to abiotic stress on the Canadian prairies: Six case studies. Can J Plant Sci **93**, 375-385.

Burke, M. J., L. V. Gusta, H. A. Quamme, C. J. Weiser and P. H. Li, 1976: Freezing and Injury in Plants. Annu Rev Plant Phys **27**, 507-528.

Canada Department of Agriculture and W. G. McGregor, 1953: Varieties of linseed flax. In: D. o. Agriculture ed.

Casa, R., G. Russell, B. Lo Cascio and F. Rossini, 1999: Environmental effects on linseed (Linum usitatissimum L.) yield and growth of flax at different stand densities. Eur J Agron **11**, 267-278.

Chang, T.-T., C.-C. Li and B. S. Vergara, 1969: Component analysis of duration from seeding to heading in rice by the basic vegetative phase and the photoperiod-sensitive phase. Euphytica **18**, 79-91.

Cheon, K.-S., A. Nakatsuka and N. Kobayashi, 2011: Isolation and expression pattern of genes related to flower initiation in the evergreen azalea, Rhododendron × pulchrum 'Oomurasaki'. Scientia Horticulturae **130**, 906-912.

Collinson, S. T., R. H. Ellis, R. J. Summerfield and E. H. Roberts, 1992: Durations of the Photoperiod-Sensitive and Photoperiod-Insensitive Phases of Development to Flowering in 4 Cultivars of Rice (Oryza-Sativa L). Ann Bot-London **70**, 339-346.

De Decker, M. M., 2007: Studies of DNA methylation and flowering time genes in early-flowering flax (Linum usitatissimum L.) lines induced by 5-azacytidine. (Master's thesis, University of Waterloo, Wateloo). Retrieved from http://hdl.handle.net/10012/3302.

Diederichsen, A. and K. Richards, 2003: Cultivated flax and the genus Linum L.: Taxonomy and germplasm conservation. Flax: the genus Linum..((Medicinal and aromatic plants: industrial profiles **34**.

Domantovich, A. V., V. A. Koshkin, N. B. Brutch and Matvienko, II, 2012: Investigation of photoperiod sensitivity of Linum usitatissimum L. Lines and effect of short-day conditions on their economically valuable traits. Russian Agricultural Sciences **38**, 173-177.

Doyle, M. R., S. J. Davis, R. M. Bastow, H. G. McWatters, L. Kozma-Bognar, F. Nagy, A. J. Millar and R. M. Amasino, 2002: The ELF4 gene controls circadian rhythms and flowering time in Arabidopsis thaliana. Nature **419**, 74-77.

Ellis, R. H., S. T. Collinson, D. Hudson and W. M. Patefield, 1992: The Analysis of Reciprocal Transfer Experiments to Estimate the Durations of the Photoperiod-Sensitive and Photoperiod-Insensitive Phases of Plant Development - an Example in Soya Bean. Ann Bot-London **70**, 87-92.

Ellis, R. H., A. Qi, P. Q. Craufurd, R. J. Summerfield and E. H. Roberts, 1997: Effects of photoperiod, temperature and asynchrony between thermoperiod and photoperiod on development to panicle initiation in sorghum. Ann Bot-London **79**, 169-178.

Endo, M., H. Shimizu, M. A. Nohales, T. Araki and S. A. Kay, 2014: Tissue-specific clocks in Arabidopsis show asymmetric coupling. Nature **515**, 419-422.

FAO, 2014: Linseed Production of Canada and Worldwide. **2014**.

Fieldes, M. A., 1994: Heritable Effects of 5-Azacytidine Treatments on the Growth and Development of Flax (Linum-Usitatissimum) Genotrophs and Genotypes. Genome **37**, 1-11.

Fieldes, M. A. and L. M. Amyot, 1999: Epigenetic control of early flowering in flax lines induced by 5-azacytidine applied to germinating seed. J Hered **90**, 199-206.

Fieldes, M. A. and C. G. Harvey, 2004: Differences in developmental programming and node number at flowering in the 5-azacytidine-induced early flowering flax lines and their controls. Int J Plant Sci **165**, 695-706.

Fieldes, M. A., S. M. Schaeffer, M. J. Krech and J. C. L. Brown, 2005: DNA hypomethylation in 5-azacytidine-induced early-flowering lines of flax. Theor Appl Genet **111**, 136-149.

Finnegan, E. J., W. J. Peacock and E. S. Dennis, 1996: Reduced DNA methylation in Arabidopsis thaliana results in abnormal plant development. P Natl Acad Sci USA **93**, 8449-8454.

Finnegan, E. J., W. J. Peacock and E. S. Dennis, 2000: DNA methylation, a key regulator of plant development and other processes. Curr Opin Genet Dev **10**, 217-223.

Furner, I. J., M. A. Sheikh and C. E. Collett, 1998: Gene silencing and homology-dependent gene silencing in Arabidopsis: Genetic modifiers and DNA methylation. Genetics **149**, 651-662.

Genger, R. K., W. J. Peacock, E. S. Dennis and E. J. Finnegan, 2003: Opposing effects of reduced DNA methylation on flowering time in Arabidopsis thaliana. Planta **216**, 461-466.

Government of Canada. (2010, 2013-01-09). "Agriculture Interactive Maps." Retrieved September 4, 2014, from http://atlas.agr.gc.ca/agmaf/index_eng.html -context=phz-zrp_en.xml&extent=-6389504.2960536,-789910.99999995,6917266.2960536,3937308.6695186&layers=place37M,place25M,place15M,place5M,place1M,place500K,place250K.

Government of Canada, C. F. I. A. P. P. P. D., Office of Plant Breeders' Rights. (2008, 2014-07-24). "Canadian Food Inspection Agency - CDC Sorrel." Retrieved September 04, 2014, from http://www.inspection.gc.ca/english/plaveg/pbrpov/cropreport/fla/app00005695e.shtml.

Government of Canada, C. F. I. A. P. P. P. D., Office of Plant Breeders' Rights. (2009, 2014-07-24). "Canadian Food Inspection Agency - Prairie Thunder." 2014, from http://www.inspection.gc.ca/english/plaveg/pbrpov/cropreport/fla/app00006344e.shtml.

Heslop-Harrison, J. S., 1990: Gene-Expression and Parental Dominance in Hybrid Plants. Development, 21-&.

Hopkins, W. G. (2006). "Plant development." from http://site.ebrary.com/id/10283887.

House, M. A., 2010: Molecular Studies of 5-azacytidine-induced Early-flowering Lines of Flax.

Huis, R., S. Hawkins and G. Neutelings, 2010: Selection of reference genes for quantitative gene expression normalization in flax (Linum usitatissimum L.). Bmc Plant Biol **10**.

Jackson, S. D., 2009: Plant responses to photoperiod. New Phytol **181**, 517-531.

Janoušek, B., J. Široký and B. Vyskot, 1996: Epigenetic control of sexual phenotype in a dioecious plant, Melandrium album. Mol Gen Genet **250**, 483-490.

Jhala, A. J. and L. M. Hall, 2010: Flax (Linum usitatissimum L.): Current uses and future applications. Aust J Basic Appl Sci **4**, 4304-4312.

Jones, P., 1984: Gene Activation by 5-Azacytidine. In: A. Razin, H. Cedar and A. Riggs eds. *DNA Methylation. Series; Springer Series in Molecular Biology, pp.* 165-187. Springer New York.

Jung, C. and A. E. Muller, 2009: Flowering time control and applications in plant breeding. Trends Plant Sci **14**, 563-573.

Kikis, E. A., R. Khanna and P. H. Quail, 2005: ELF4 is a phytochrome-regulated component of a negativefeedback loop involving the central oscillator components CCA1 and LHY. Plant J **44**, 300-313.

King, G. J., 1995: Morphological Development in Brassica-Oleracea Is Modulated by in-Vivo Treatment with 5-Azacytidine. J Hortic Sci **70**, 333-342.

Levy, Y. Y. and C. Dean, 1998: The transition to flowering. Plant Cell **10**, 1973-1989.

Lin, C. S. and G. Poushinsky, 1983: A Modified Augmented Design for an Early Stage of Plant-Selection Involving a Large Number of Test Lines without Replication. Biometrics **39**, 553-561.

Lin, C. S. and G. Poushinsky, 1985: A Modified Augmented Design (Type-2) for Rectangular Plots. Can J Plant Sci **65**, 743-749.

Lin, C. S. and H. D. Voldeng, 1989: Efficiency of Type-2 Modified Augmented Designs in Soybean Variety Trials. Agron J **81**, 512-517.

Lin, L., H. Allemekinders, A. Dansby, L. Campbell, S. Durance-Tod, A. Berger and P. J. H. Jones, 2013: Evidence of health benefits of canola oil. Nutrition reviews **71**, 370-385.

Livak, K. J. and T. D. Schmittgen, 2001: Analysis of relative gene expression data using real-time quantitative PCR and the 2(T)(-Delta Delta C) method. Methods **25**, 402-408.

Lowcock, E. C., M. Cotterchio and B. A. Boucher, 2013: Consumption of flaxseed, a rich source of lignans, is associated with reduced breast cancer risk. Cancer Causes & Control **24**, 813-816.

Manggoel, W. and M. I. Uguru, 2012: Evidence of Maternal Effect on the Inheritance of Flowering Time in Cowpea (Vigna unguiculata (L.) Walp.). International Journal of Plant Breeding & Genetics **6**.

Matzke, M. A. and A. J. M. Matzke, 1998: Epigenetic silencing of plant transgenes as a consequence of diverse cellular defence responses. Cellular and Molecular Life Sciences CMLS **54**, 94-103.

Mayland, H. F. and J. W. Cary, 1970: Frost and chilling injury to growing plants. Advances in Agronomy **22**, 203-234.

McClung, A. J., III, 2006a: Techniques for dynamic maneuvering of hexapedal legged robots*. pp.* 216 p. Stanford University.

McClung, C. R., 2006b: Plant circadian rhythms. The Plant Cell Online **18**, 792-803.

McWatters, H. G., E. Kolmos, A. Hall, M. R. Doyle, R. M. Amasino, P. Gyula, F. Nagy, A. J. Millar and S. J. Davis, 2007: ELF4 is required for oscillatory properties of the circadian clock. Plant Physiol **144**, 391-401.

Messina, M. J., 1999: Legumes and soybeans: overview of their nutritional profiles and health effects. The American journal of clinical nutrition **70**, 439s-450s.

Muir, A. D. and N. D. Westcott, 2003: Flax: the genus Linum. CRC Press.

Munir, M., P. Hadley, J. Carew, M. Zubair, S. Adams, S. B. Hussain, J. U. D. Baloch, N. Hussain and M. Amanullah, 2010: An Appraisal of the Use of Reciprocal Transfer Experiments: Assessing the Stages of Photoperiod Sensitivity in Antirrhinum Majus L. Pak J Bot **42**, 1931-1940.

Nichterlein, K. and R. Horn, 2005: Haploids in crop improvement II. Springer Berlin Heidelberg.

Pan, R., 2001a: Growth physiology of plant. In: R. Pan, X. Wang and N. Li eds. *Plant Physiol. pp.* 219-240. Higher Education Press, Beijing.

Pan, R., 2001b: Photomorphogenesis. In: R. Pan, X. Wang and N. Li eds. *Plant Physiol. pp.* 206-218. Higher Education Press, Beijing.

Pan, R., 2001c: Plant Reproductive Physiology. In: R. Pan, X. Wang and N. Li eds. *Plant Physiol. pp.* 245-249. Higher Education Press, Beijing.

Pareek, A., S. K. Sopory and H. J. Bohnert, 2010: Abiotic stress adaptation in plants: physiological, molecular and genomic foundation. Springer, Dordrecht.

Phelps, S., 2004: Frost damage to crops. In: G. o. S. Ministry of Agriculture ed.

Richards, E. J., 1997: DNA methylation and plant development. Trends Genet **13**, 319-323.

Rowland, G. G., Y. A. Hormis and K. Y. Rashid, 2002: CDC Bethune Flax. Can J Plant Sci **82**, 101-102.

Rowland, G. G., E. O. Kenaschuk and R. S. Bhatty, 1990: Flanders Flax. Can J Plant Sci **70**, 543-544.

Sano, H., I. Kamada, S. Youssefian, M. Katsumi and H. Wabiko, 1990: A Single Treatment of Rice Seedlings with 5-Azacytidine Induces Heritable Dwarfism and Undermethylation of Genomic DNA. Mol Gen Genet **220**, 441-447.

Saskatchewan Ministry of Agriculture, G. o. S., 2008: Effect Of Fall Frost On Seed Quality.

Saskatchewan Ministry of Agriculture, G. o. S., 2014: Varieties of Grain Crops 2014. In: G. o. S. Ministry of Agriculture ed.

Scheid, O. M., A. V. Probst, K. Afsar and J. Paszkowski, 2002: Two regulatory levels of transcriptional gene silencing in Arabidopsis. P Natl Acad Sci USA **99**, 13659-13662.

Serraj, R., L. Krishnamurthy, J. Kashiwagi, J. Kumar, S. Chandra and J. H. Crouch, 2004: Variation in root traits of chickpea (< i> Cicer arietinum</i> L.) grown under terminal drought. Field Crop Res **88**, 115-127.

Singh, B. P., 2010: Industrial crops and uses. CABI, Wallingford, Oxfordshire, UK; Cambridge, MA.

Sizov, I. A., 1955: Len.[Flax]. Sel'chozgiz, Moscow, Leningrad.

Song, Y. H., S. Ito and T. Imaizumi, 2013: Flowering time regulation: photoperiod and temperature sensing in leaves. Trends Plant Sci **18**, 575-583.

Sosulski, F. W. and R. F. Gore, 1964: THE EFFECT OF PHOTOPERIOD AND TEMPERATURE ON THE CHARACTERISTICS OF FLAXSEED OIL. Can J Plant Sci **44**, 381-382.

Soto-Cerda, B. J., S. Duguid, H. Booker, G. Rowland, A. Diederichsen and S. Cloutier, 2014: Genomic regions underlying agronomic traits in linseed (Linum usitatissimum L.) as revealed by association mapping. Journal of integrative plant biology **56**, 75-87.

Staiger, D., J. Shin, M. Johansson and S. J. Davis, 2013: The circadian clock goes genomic. Genome biology **14**, 208.

Statistics Canada. (2014, 2014-10-03). "Field and special crops (Seeded area)." 2014, from http://www.statcan.gc.ca/tables-tableaux/sum-som/l01/cst01/prim11a-eng.htm.

Takeno, K., 2010: Epigenetic regulation of photoperiodic flowering. Plant signaling & behavior **5**, 788.

Thomas, B. and D. Vince-Prue, 1996: Photoperiodism in plants. Academic Press.

Vyskot, B., B. Koukalova, A. Kovařík, L. Sachambula, D. Reynolds and M. Bezděk, 1995: Meiotic Transmission of a Hypomethylated Repetitive DNA Family in Tobacco. Theor Appl Genet **91**, 659-664.

Wang, Y., J. F. Wu, N. Nakamichi, H. Sakakibara, H. G. Nam and S. H. Wu, 2011: LIGHT-REGULATED WD1 and PSEUDO-RESPONSE REGULATOR9 form a positive feedback regulatory loop in the Arabidopsis circadian clock. Plant Cell **23**, 486-498.

Wang, Z. C., M. C. Acock and B. Acock, 1997: Photoperiod sensitivity during flower development of opium poppy (Papaver somniferum L). Ann Bot-London **79**, 129-132.

Yin, X. Y., 2008: Analysis of reciprocal-transfer experiments to estimate the length of phases having different responses to temperature. Ann Bot-London **101**, 603-611.

Yong, W. D., K. Chong, Z. H. Xu, K. H. Tan and Z. Q. Zhu, 2000: Gene control of flowering time in higher plants. Chinese Sci Bull **45**, 1633-1642.

You, F. M., S. D. Duguid, D. Thambugala and S. Cloutier, 2013: Statistical analysis and field evaluation of the type 2 modified augmented design (MAD) in phenotyping of flax ('Linum usitatissimum') germplasms in multiple environments. Australian Journal of Crop Science **7**, 1789.

Zhang, T., 2013: Characterizing the Flax Core Collection for Earliness and Canopy Traits (Master's thesis, University of Saskatchewan, Saskatoon). Retrieved from http://hdl.handle.net/10388/ETD-2013-04-983.

Zhang, T., E. G. Lamb, B. Soto-Cerda, S. Duguid, S. Cloutier, G. Rowland, A. Diederichsen and H. M. Booker, 2014: Structural equation modeling of the

Canadian flax (Linum usitatissimum L.) core collection for multiple phenotypic traits. Can J Plant Sci **94**, 1325-1332.

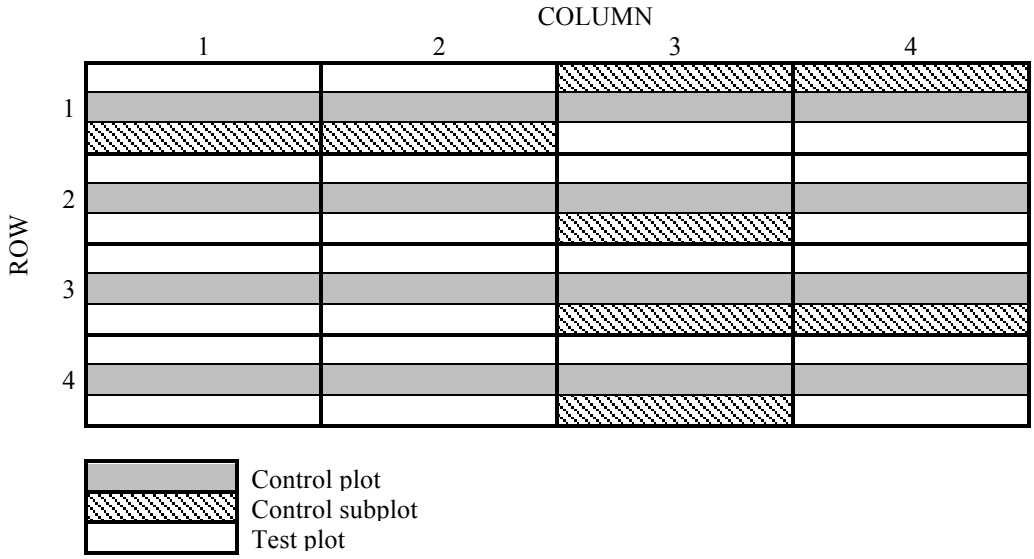
Appendices

Appendix I : Levene's Test for Homogeneity of Variance between two rounds of the experiments for each cultivar or genotype for Chapter 3.

	Source	DF	Sum of Square	Mean of Square	F value	Pr > F
CDC Sorrel	Round	1	9057.4	9057.4	4.76	0.0306*
	Error	165	314070	1903.5		
CDC Bethune	Round	1	1508.1	1508.1	3.7	0.056
	Error	165	67169.5	407.1		
Flanders	Round	1	51.5077	51.5077	0.02	0.9015
	Error	165	552766	3350.1		
Prairie Thunder	Round	1	2980.9	2980.9	1.64	0.2015
	Error	166	300868	1812.5		
Royal	Round	1	12.4134	12.4134	0.01	0.9351
	Error	166	309601	1865.1		
RE1	Round	1	3636.9	3636.9	2.94	0.0885
	Error	166	205691	1239.1		
RE2	Round	1	32.988	32.988	1.34	0.2486
	Error	164	4034.7	24.6019		
RE3	Round	1	270.3	270.3	2.42	0.122
	Error	165	18461.6	111.9		

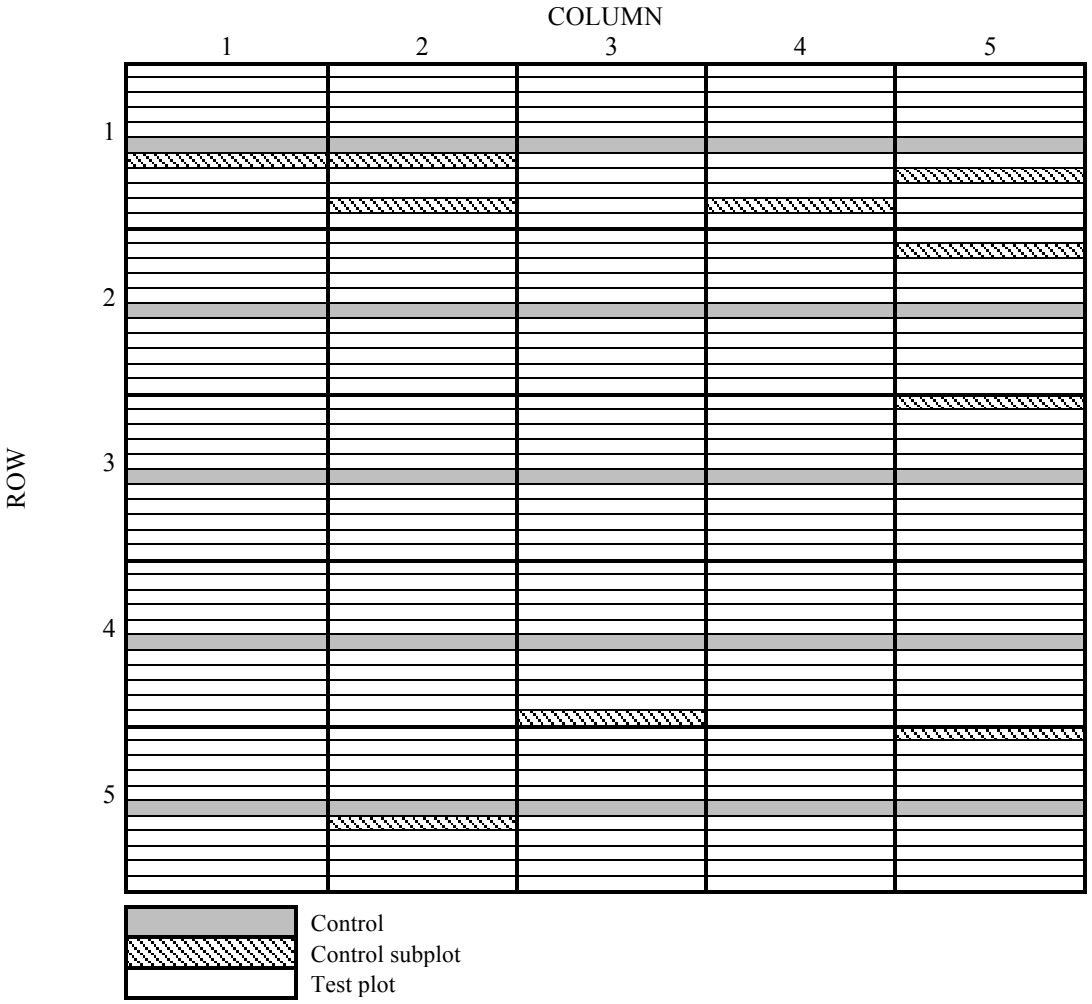
According to the Levene's test, for CDC Sorrel the variances were not equal across the two rounds of the experiment (p=0.03). Even so data were combined for analysis.

Appendix II: A diagram of field layout of F_2 field test.



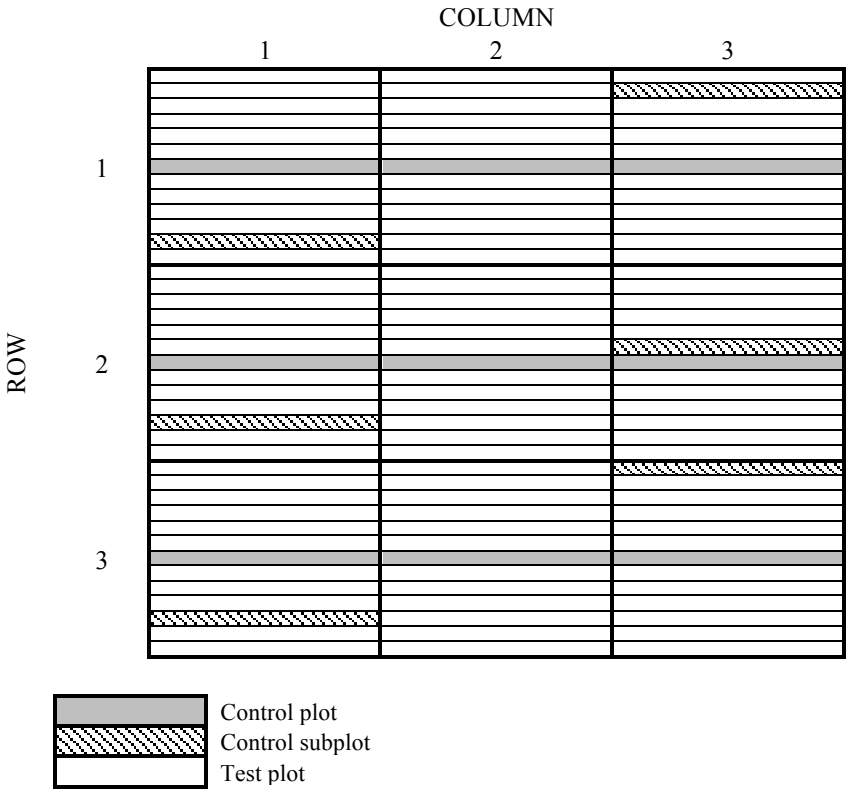
Plots were arranged in 4x4 grids. Each whole plot (indicated by a thicker border) was split into three subplots with a plot control in the centre subplot. Control plots included CDC Sorrel, RE1, RE2 and RE3, indicated with grey boxes. Control subplots including the same cultivars/genotypes (CDC Sorrel, RE1, RE2 and RE3), indicated with the striped boxes, and were randomly allocated in the selected eight whole plots. A total of 16 rows were randomly distributed in the remaining subplots (test plots).

Appendix III: A diagram of field layout of F_3 field test.



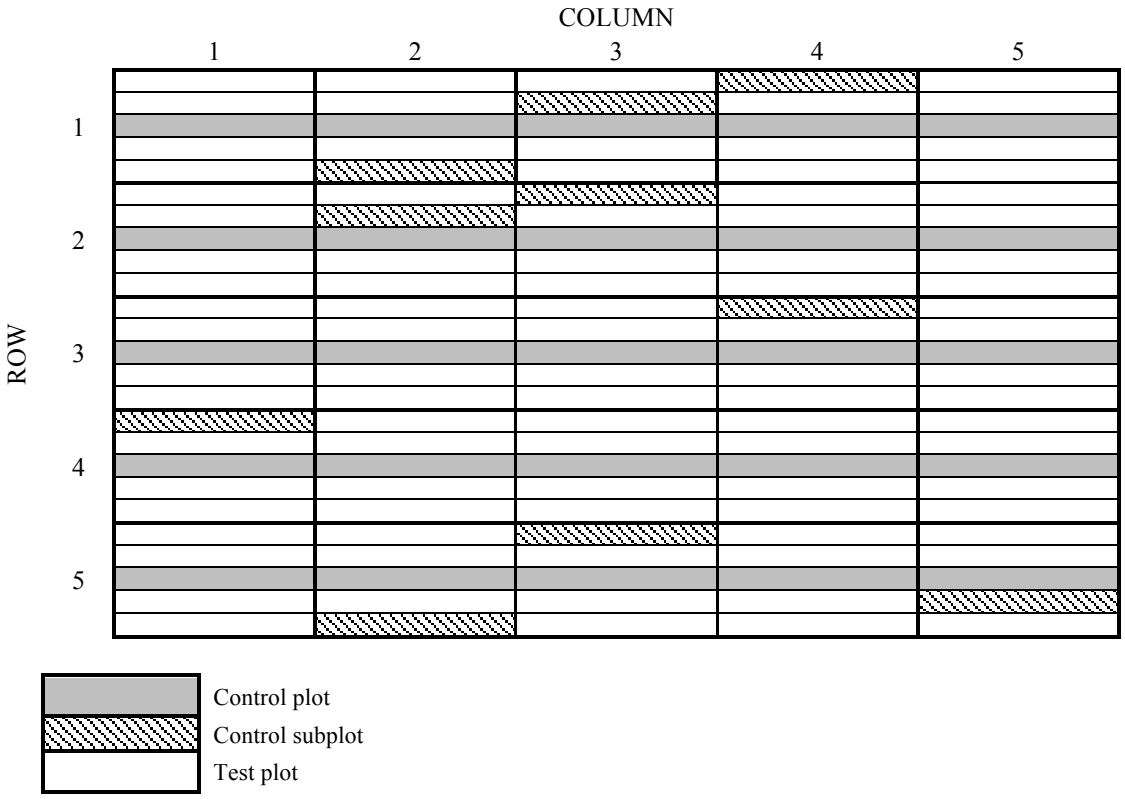
Plots were arranged in 5x5 grids. Each whole plot (indicated with thicker border) was split into 11 subplots with a plot control in the centre subplot. Control plots included CDC Sorrel, Royal, RE1, RE2 and RE3, indicated with grey boxes. Control subplots including the same cultivars and genotypes (CDC Sorrel, Royal, RE1, RE2 and RE3), indicated by striped boxes and were randomly allocated in the selected ten whole plots. A total of 229 test genotypes were randomly distributed in the remaining subplots (test plots).

Appendix IV: A diagram of field layout of M_2 field test.



Plots were arranged in 3x3 grids. Each whole plot (indicated with thicker border) was split into 13 subplots with a plot control in the centre subplot. Control plots included CDC Sorrel, Royal, RE1, RE2 and RE3, indicated by grey boxes. Control subplots including cultivars and genotypes (CDC Sorrel, Royal, RE1, RE2 and RE3), indicated by striped boxes, and were randomly allocated in the selected six whole plots. 98 test rows were randomly distributed in the remaining subplots (test plots).

Appendix V: A diagram of field layout of M_3 field test.



Plots were arranged in 5x5 grids. Each whole plot (indicated with thicker border) was split into five subplots with a plot control in the centre subplot. Control plots included CDC Sorrel, Royal, RE1, RE2 and RE3, indicated by grey boxes. Control subplots including the same cultivars and genotypes (CDC Sorrel, Royal, RE1, RE2 and RE3), indicated by striped boxes, and were randomly allocated in the selected six whole plots. 78 test rows were randomly distributed in the remaining subplots (test plots).

Appendix VI: ANOVA of DTF, DTM, HT and TSW for plot controls and subplot controls in F_2 population.

Source	DTF				DTM				HT (cm)				TSW (g)			
	DF	SS	MS	F	DF	SS	MS	F	DF	SS	MS	F	DF	SS	MS	F
Analysis of plot control																
Controls	3	252.75	84.25	19.82**	3	398.19	132.73	28.57**	3	1517.25	505.75	13.37**	3	3.69	1.23	2.00
Rows(R)	3	142.25	47.42	11.16**	3	467.19	155.73	33.52**	3	145.25	48.42	1.28	3	1.85	0.62	1.00
Columns(C)	3	7.25	2.42	0.57	3	31.69	10.56	2.27	3	136.25	45.42	1.20	3	0.93	0.31	0.50
Plot error	6	25.5	4.25	0.62	6	27.88	4.65	0.13	6	227	37.83	0.99	6	3.70	0.62	1.11
Analysis of subplot controls																
Sub-controls	3	523.25	174.42	25.37**	3	366	122	3.40	3	1651.5	550.5	14.41**	3	3.64	1.21	2.18*
Subplot error	12	82.5	6.88		12	431	35.92		12	458.5	38.21		12	6.68	0.56	

Appendix VII: ANOVA of DTF, DTM, HT and TSW for plot controls and subplot controls in F_3 population.

Source	DTF				DTM				Height (cm)				TSW (g)			
	DF	SS	MS	F	DF	SS	MS	F	DF	SS	MS	F	DF	SS	MS	F
Analysis of plot control																
Controls	4	494.16	123.54	35.23**	4	289.04	72.26	12.84**	4	1794.64	448.66	107.00**	4	3.09	0.77	49.66**
Rows(R)	4	19.76	4.94	1.41	4	30.64	7.66	1.36	4	28.64	7.16	1.71	4	0.09	0.02	1.38
Columns(C)	4	137.36	34.34	9.79**	4	80.24	20.06	3.57*	4	42.64	10.66	2.54	4	0.20	0.05	3.23**
Plot error	12	42.08	3.51	0.39	12	67.52	5.63	0.82	12	50.32	4.19	0.24	12	0.19	0.016	0.71
Analysis of subplot controls																
Sub-controls	4	276.20	69.05	7.67**	4	275.70	68.93	9.99**	4	1226.70	306.68	17.68**	4	2.39	0.60	27.41**
Subplot error	15	135.00	9.00		15	103.50	6.90		15	260.25	17.35		15	0.33	0.02	

Appendix VIII: Hybrid population coefficient of variation (CV) of unadjusted (unadj) and adjusted values by Method 1 (M1) and Method 3 (M3) for plot and subplot controls and relative efficiencies (RE) measured by error variance of all controls.

Year	Trait	CV(%) for plot controls				CV(%) for subplot controls				RE			Best adjustment by	
		Unadj	M1	M3	M1+M3	Unadj	M1	M3	M1+M3	M1	M3	M1+M3	RE	ANOVA
2012	DTF	8.59	8.59	0.24	0.24	12.95	12.08	11.68	11.68	100	348.21	348.21	M3	M1
2013	DTF	6.80	6.80	4.99	4.99	7.82	11.2	7.40	7.40	40.12	125.72	125.7	M3	M1
2012	DTM	5.77	5.77	1.57	1.57	7.65	9.12	8.14	8.14	112.31	168.44	168.44	M3	M1
2013	DTM	2.30	2.30	1.43	1.43	4.57	5.31	4.54	4.54	51.37	167.65	167.65	M3	M1
2012	HT	3.18	3.18	3.07	3.07	24.34	25.43	24.36	24.36	103.22	100.85	100.85	Un.	Un.
2013	HT	3.98	3.98	4.01	4.01	12.30	13.01	12.30	12.30	44.21	99.50	99.50	Un.	Un
2012	TSW	2.21	2.21	2.74	2.74	15.76	15.71	15.78	15.78	96.58	99.39	99.39	Un.	Un
2013	TSW	2.17	2.17	1.18	6.12	6.18	6.45	6.08	1.21	72.99	165.07	163.75	Un.	Un.

Un.: unnecessary. If there's no significant row and column effect, then no adjustment to the raw data is considered necessary.

Appendix IX: ANOVA of DTF, DTM, HT and TSW of M_2 population.

Source	DTF				DTM				Height (cm)				TSW (g)			
	DF	SS	MS	F	DF	SS	MS	F	DF	SS	MS	F	DF	SS	MS	F
Analysis of plot control																
Controls	2	80.22	40.11	90.25*	2	128.22	64.11	36.06*	2	472.89	505.75	15.31	2	0.95	0.47	3.14
Rows(R)	2	2.89	1.44	3.25	2	110.89	55.44	31.19*	2	72.22	48.42	2.34	2	0.10	0.05	0.34
Columns(C)	2	28.22	14.11	31.75*	2	10.89	5.44	3.06	2	38.22	45.42	1.24	2	0.08	0.04	0.26
Plot error	2	25.50	4.25	0.62	2	3.56	1.78	0.07	2	30.89	37.83	0.75	2	0.30	0.15	1.95
Analysis of subplot controls																
Sub-controls	2	523.25	174.42	25.37*	2	66.50	33.25	1.40	2	378.50	550.50	9.13**	2	1.23	0.62	7.96*
Subplot error	9	82.50	6.88		9	213.75	23.75		9	168.50	38.21		9	0.70	0.08	

Appendix X: ANOVA of DTF, DTM, HT and TSW of M_3 population.

Source	DTF				DTM				Height (cm)				TSW (g)			
	DF	SS	MS	F	DF	SS	MS	F	DF	SS	MS	F	DF	SS	MS	F
Analysis of plot control																
Genotype	4	351.04	87.76	29.99**	4	280.16	70.04	16.52**	4	1889.2	472.3	30.28**	4	1.44	0.36	16.66**
Rows(R)	4	34.64	8.66	2.95	4	10.96	2.74	0.65	4	95.2	23.8	1.53	4	0.15	0.04	1.70
Columns(C)	4	37.84	9.46	3.23	4	19.76	4.94	1.17	4	18.40	4.60	0.29	4	0.03	0.01	0.31
Plot error	12	35.12	2.93	0.31	12	50.88	4.24	0.71	12	187.2	15.6	0.39	12	0.26	0.02	0.76
Analysis of subplot controls																
Controls	4	103.70	25.925	2.79	4	289.30	72.33	12.12**	4	1070.30	267.58	6.71**	4	1.63	0.41	14.28**
Subplot error	15	139.50	9.30		15	89.50	5.97		15	598.50	39.90		15	0.43	0.03	

Appendix XI: Mutant population coefficient of variation (CV) of unadjusted (unadj) and adjusted values by Method 1 (M1) and Method 3 (M3) for plot and subplot controls and relative efficiencies (RE) measured by error variance of all controls.

Year	Trait	CV(%) for plot controls				CV(%) for subplot controls				RE			Best adjustment by	
		Unadj	M1	M3	M1+M3	Unadj	M1	M3	M1+M3	M1	M3	M1+M3	RE	ANOVA
2012	DTF	4.6	4.6	2.2	2.2	7.3	9.9	7.8	7.8	54.8	136.3	136.4	M1+M3	M1
2013	DTF	1.8	1.8	0.7	0.7	6.0	6.1	5.9	5.9	264.3	148.7	148.7	Un.	Un.
2012	DTM	3.6	3.6	1.4	1.4	2.9	5.4	3.1	3.1	43.1	138.3	138.3	M3	M1
2013	DTM	1.8	1.8	0.8	0.8	4.9	6.06	5.1	5.1	57.5	138.3	138.3	Un.	Un.
2012	HT	10.7	10.7	9.3	9.3	9.6	18.0	9.5	9.5	48.2	129.9	129.9	Un.	Un.
2013	HT	3.0	3.0	0.7	0.7	14.9	15.2	14.9	14.9	109.1	430.1	430.1	Un.	Un.
2012	TSW	4.17	4.17	6.18	6.09	5.49	4.14	6.14	6.14	55.34	66.2	67.03	Un.	Un
2013	TSW	2.5	2.5	1.15	1.19	5.88	6.53	5.92	5.92	52.04	185.25	181.59	Un	Un

Un.: unnecessary. If there's no significant row and column effect, then no adjustment to the raw data is considered necessary.